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GLUCAGON-LIKE PEPTIDE-1 AND GLUCAGON-LIKE PEPTIDE-1 ANALOGS NANOTECHNOLOGY-BASED SYSTEMS FOR PREVENTION AND THERAPY OF DIABETES

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“Quando alimentamos mais a nossa coragem que os nossos medos

passamos a derrubar muros e a construir pontes”

(Lígia Guerra)

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ABSTRACT

Type 2 diabetes *mellitus* (T2DM) is one of the most prevalent diseases worldwide. It is primary associated with impaired insulin secretion by the pancreatic β-cells and/or insulin resistance. Current treatments are often associated with variations of normalizing glycemic levels, off-target effects and do not significantly impact disease progression. New therapies are, therefore, urgently needed to overcome this social burden.

Incretin based treatments, especially using glucagon-like peptide-1 (GLP-1), an incretin hormone which is a glucose-dependent insulinotropic peptide, are now widely investigated and used to manage T2DM. The oral route is, by far, the most desirable way for drug delivery, but when it comes to peptides and proteins, it is traditionally challenging and frequently compromised using conventional dosage form approaches. This is due to the main barriers encountered along the gastrointestinal tract, namely the enzymatic and chemical degradations, mucus layer and the epithelial barrier. Thus, the oral administration of GLP-1 is still a huge challenge in the pharmaceutical field, requiring administration by parenteral routes, which severely decrease patient adherence to the treatment.

Recent developments in materials science and nanomedicine, and specific surface functionalization strategies, are providing new tools for the rational design of precisely engineered drug delivery systems. Particular interest has been paid to the exploitation of tailored nanomaterials for the administration of peptides and proteins by the oral route. Using such materials for producing nanoparticulate systems is a promising approach to obtain advanced drug delivery systems capable of providing stable and biocompatible environments for these drugs and allowing for a targeted delivery of the associated biopharmaceuticals.

The main aim of this thesis was to develop safe particulate-based GLP-1 carrier systems and evaluate their efficiency in increasing GLP-1 oral bioavailability. The first part of this was focused on selecting the most adequate material in terms of particle size and surface charge, GLP-1 association efficiency (AE), drug release and interaction with intestinal cell lines. Poly(lactide-co-glycolide) (PLGA), Witepsol E85 lipid (solid lipid nanoparticles, SLN) and porous silicon (PSi) nanoparticles were tested. All the particles presented a size approximately around 200 nm with negative zeta potential and narow polydispersity index. Due to their negative charge, the interactions with the intestinal cells were expected to be minimal. Chitosan (CS), a polycationic and partially mucoadhesive biopolymer, with known permeation enhancing effect, was used to modify the surface of the nanoparticles. When coated with CS, nanoparticles size increased and there was an

inversion of the zeta potential to positive values. CS functionalized PLGA and PSi nanoparticles presented the best properties, namely mucoadhesion, closer interaction with intestinal cell lines and an AE with GLP-1 of 60% and 85%, respectively.

A complex multistage delivery system was further developed to improve GLP-1 delivery. In order to enhance the transcellular permeability of GLP-1, a cell penetrating peptide (CPP) was further attached to the nanoparticles surface. In addition, the nanoparticles were subsequently encapsulated into pH-sensitive hydroxypropylmethylcellulose acetylsuccinate (HPMC-AS)-based microparticles of size of *ca.* 60 µm, using a microfluidics technique, to protect the nanoparticles and the premature GLP-1 release from the very low gastric pH. HPMC-AS dissolves only at pH ≥ 6.0, which allows controlling the release of GLP-1 until the particles reach the small intestine. A small molecule drug, able to inhibit the dipeptidyl peptidase 4 (DPP4) enzyme, responsible to cleave and hence inactivate GLP-1 *in vivo*, was simultenously loaded into HPMC-AS microparticles (AE ≈ 20%). The loading degree of GLP-1 was of 0.030 ± 0.007% and 0.730 ± 0.001 % for the PLGA and PSi systems encapsulated into HPMC-AS, respectively, and for iDPP4 was of 1.00 ± 0.01% and 1.26 ± 0.01%, respectively. This multistage platform showed a pH-dependency, with regular and smooth particle shape and surface morphology at acidic pH, thus protecting nanoparticles and GLP-1 in acidic conditions. In turn, at more neutral intestinal pH, HPMC-AS microparticles disaggregate, thereby releasing the CS-CPP conjugated nanoparticles and providing stronger interactions with the intestinal cells. CS-CPP conjugated PLGA and PSi nanoparticles presented a 5.6 and 1.3-fold increase in the interaction with the intestinal cells, compared to the unmodified nanoparticles, respectively. The presence of the DPP4 inhibitor decreased the DPP4 activity over 20-fold and increased GLP-1 permeability across a triple co-culture intestinal monolayer, with a *ca.* 5-fold increase for the PLGA systems and a *ca.* 1.5-fold increase for the PSi ones.

When tested *in vivo* after oral administration in a non-obese T2DM rat model, the HPMC-AS microparticles with CS-CPP conjugated PLGA nanoparticles, containing the combination of GLP-1 and DPP4 inhibitor, resulted in a decreased in the hyperglycemia in a sustained and prolonged manner. After oral administration of the system, blood glucose levels decreased *ca.* 45%, from 4h until at least 8h after administration, presenting half of the glucose area under the curve when compared to the control (GLP-1 + DPP4 inhibitor in solution). The insulin plasmatic levels were also increased 6h after particles oral administration as well as the pancreatic insulin content.

Overall, a multistage composite system was developed for oral dual delivery of GLP-1 and DPP4 inhibitor. This platform showed to be successful in overcoming the barriers along the gastrointestinal tract and increasing the GLP-1 oral bioavailabilty, thus showing clinical

potential as an oral peptide and protein delivery system for T2DM therapy, and, potentially, for other biopharmaceutical-based therapies.

RESUMO

A diabetes *mellitus* tipo 2 (T2DM) é uma das doenças mais prevalentes em todo o mundo, caracterizada pela incorreta produção de insulina pelas células β do pâncreas e/ou pela resistência do organismo na utilização da insulina produzida. As terapias usadas atualmente para o controlo da sintomatologia da T2DM estão, na maioria das vezes, associadas a múltiplas variações dos níveis de glucose e a efeitos secundários, não tendo, por isso, um grande impacto na sua progressão. Assim, novas terapias são necessárias de forma a controlar a sintomatologia desta doença com maior efetividade, e racionalizar os custos associados ao seu tratamento.

Terapias com base em incretinas, especialmente o *glucagon-like peptide-1* (GLP-1), uma hormona com efeito insulinotrópico dependente de glucose, têm sido investigados e utilizados no controlo da T2DM. A via de administração oral é, sem dúvida, a preferida para a administração de fármacos, mas quando estes são de origem proteica, como é o caso do GLP-1, a sua atividade está normalmente comprometida quando formulados em formas farmacêuticas convencionais. Assim, a administração de GLP-1 é realizada por vias parentéricas invasivas, o que diminui drasticamente a adesão dos pacientes à terapia.

O recente aumento do conhecimento da ciência dos materiais, e em especial na funcionalização da sua superfície, tem proporcionado novas ferramentas para o desenvolvimento de sistemas de elevada precisão para a administração de fármacos, com particular interesse para a administração de péptidos e proteínas por via oral. O uso desses biomateriais para a produção de sistemas multiparticulares é uma abordagem cada vez mais comum, obtendo-se assim sistemas mais inteligentes de administração de fármacos, capazes de fornecer um microambiente estável e biocompatível para o fármaco e permitindo uma administração mais orientada.

O principal objetivo desta tese foi desenvolver sistemas particulares para administração oral de GLP-1, que aumentassem a sua biodisponibilidade. A primeira etapa do trabalho foi escolher, entre diferentes materiais como o ácido poli(láctico-co-glicólico) (PLGA), lípido Witepsol E85 e sílica porosa (PSi), os mais promissores relativamente ao tamanho das partículas, à sua carga superficial, à eficiência de associação (AE), libertação do GLP-1 e interação com células intestinais. Todas as nanopartículas apresentaram tamanhos próximos dos 200 nm, com índice de polidispersão baixos e carga superficial negativa. Devido a esta carga, as interações com as células intestinais são expectavelmente reduzidas. Assim, os sistemas particulares foram modificados com quitosano (CS), um

polímero policatiónico e parcialmente mucoadesivo, conhecido também por aumentar a permeabilidade celular. Quando modificadas com o CS, o tamanho médio das partículas aumentou e a carga inverteu para valores positivos. As nanopartículas de PLGA e PSi, ambas modificadas com CS, foram as que apresentaram melhores resultados, com propriedades de mucoadesão e maior interação com as linhas celulares do que os sistemas não-modificados. As suas AE foram de 60% e 85%, respetivamente.

Numa etapa posterior, os sistemas foram melhorados para que conseguissem resistir e ultrapassar todas as barreiras encontradas ao longo do trato gastrointestinal. Para isso, foi feita uma funcionalização com um péptido penetrador de células (CPP) na sua superfície, com a finalidade de melhorar a permeabilidade transcelular de GLP-1. As nanopartículas foram depois encapsuladas em micropartículas constituídas por um polímero sensível ao pH, o hidroxipropilmetilcelulose acetil succinato (HPMC-AS), através da técnica de microfluidos, dando origem a micropartículas com diâmetro ≈ 60 µm. Esta microencapsulação das nanoparticulas foi realizada para que permitisse uma proteção das nanoparticulas no pH muito baixo do estômago. O HPMC-AS, que se dissolve apenas a um pH ≥ 6.0, permite controlar a libertação das nanoparticulas e do GLP-1 até que as micropartículas atinjam o intestino. A este polímero sensível ao pH foi simultaneamente adicionado um fármaco de baixo peso molecular, inibidor da enzima dipeptidyl-peptidase 4 (DPP4), responsável por clivar e inativar o GLP-1 *in vivo* (AE ≈ 20%). A dosagem de GLP-1 para os sistemas the PLGA e PSi encapsulados em HPMC-AS foram de 0.030 ± 0.007 % e 0.730 ± 0.001 %, respetivamente, e a dosagem do iDPP4 foi de 1 ± 0.01 % e 1.26 ± 0.01%, respectivamente. Estas micropartículas apresentavam uma superfície lisa e morfologia regular, e demonstraram ter um perfil de libertação pH-dependente, retendo o GLP-1 no seu interior a pH ácido. Por sua vez, a pH intestinal, o polímero dissolveu-se e as nanopartículas libertadas apresentaram maior interação com as células intestinais em comparação com os sistemas não-modificados. Isto mostra que a encapsulação das nanoparticulas nas micropartículas de HPMC-AS utilizando a técnica de microfluidos foi eficaz e que as nanopartículas foram protegidas em condições ácidas. As nanopartículas de PLGA e PSi modificadas com CS-CPP apresentaram 5.6 e 1.3 vezes mais interação com as células intestinais, relativamente às nanopartículas não-modificadas, respetivamente. A presença do inibidor da DPP4 diminuiu a actividade enzimática da DPP4 mais de 20 vezes e aumentou também a permeabilidade de GLP-1 através de um modelo triplo intestinal em ≈ 5 vezes para os sistemas contendo PLGA e ≈ 1.5 vezes para os sistemas contendo PSi, em comparação com sistemas sem o inibidor.

Quando testados *in vivo,* num modelo de rato de T2DM não-obesos, após administração oral, as micropartículas de HPMC-AS com as nanopartículas de PLGA modificadas com CS-CPP, contendo GLP-1 e o inibidor da DPP4, originaram uma

diminuição da hiperglicemia de uma forma sustentada e prolongada. Com a administração oral do sistema, os níveis de glicose no sangue foram reduzidos em 45%, pelo menos das 4 h às 8 h após administração, apresentando ainda metade da área sob a curva de glucose quando comparado com o controlo (solução de GLP-1 + inibidor da DPP4). Também os valores plasmáticos de insulina aumentarem 6 h após a administração das partículas assim como o conteúdo pancreático de insulina.

Em conclusão, uma plataforma para a co-administração oral do GLP-1 e do inibidor da DPP4 foi desenvolvida, aumentando a biodisponibilidade oral do GLP-1 quando administrada oralmente. Esta plataforma mostrou ter sucesso em resistir e ultrapassar as barreiras do trato gastrointestinal, mostrando ter potencial para o seu uso clínico como sistemas de entrega oral de péptidos e proteínas para a terapia da T2DM e, eventualmente, para outras terapias dependentes de biofármacos.

ACRONYMS AND ABBREVIATIONS LIST

AFR	Aerosol flow reactor
ATP	Adenosine tyrosine phosphatase
AUC	Area under the curve
CPP	Cell penetrating peptide
CS	Chitosan
DAPI	4', 6-diamidino-2-phenylindole, dihydrochloride
DMEM	Dulbecco's Modified Eagle's Medium
DPP4	Dipeptidyl peptidase 4
EDC	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
IC_{50}	Half maximal inhibitory concentration
FaSSIF	Fasted state simulated intestinal fluid
FITC	Fluorescein isothiocyanate
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 receptor
HBSS	Hank's balanced salt solution
HEPES	2-(4-(2-Hydroxyethyl) piperazin-1-yl) ethanesulfonic acid
HPLC	High performance liquid chromatography
H-PLGA	Multisystem constituted by poly(lactic-co-glycolic acid) nanoparticles modified with chitosan and cell penetrating peptide encapsulated into hydroxypropyl methylcellulose acetate succinate loaded with glucagon-like peptide-1 and dipeptidyl peptidase 4 inhibitor
HPMC-AS	Hydroxypropyl methylcellulose acetate succinate
H-PSi	Multisystem constituted by porous silicon nanoparticles modified with chitosan and cell penetrating peptide encapsulated into hydroxypropyl methylcellulose acetate succinate loaded with glucagon-like peptide-1 and dipeptidyl peptidase 4 inhibitor
iDPP4	Dipeptidyl peptidase 4 inhibitor
i.p.	Intraperitoneal
kDa	kiloDalton

LD_{50}	Letal dose 50%
MES	2-(N-morpholino) ethanesulfonic acid
NHS	N-hydroxysuccinimide
PAA	Poly(acrylic acid)
PC 1/3	Prohormone convertase 1/3
PC 2	Prohormone convertase 2
PEG	Polyethylene glycol
PLGA	Poly(lactic-co-glycolic acid)
PLGA+CS	Chitosan-coated poly(lactic-co-glycolic acid)
PLGA+CS-CPP	Cell penetrating peptide and chitosan modified poly(lactic-co-glycolic acid)
PSi	Porous silicon
PSi+CS	Chitosan-coated porous silicon
PSi+CS-CPP	Cell penetrating peptide and chitosan modified porous silicon
PVA	Poly(vinyl alcohol)
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SD	Standard deviation
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SLN	Solid lipid nanoparticles
SLN+CS	Chitosan-coated solid lipid nanoparticles
STZ	Streptozotocin
T2DM	Type 2 diabetes *mellitus*
UnTHCPSi	Undecylenic acid modified thermally hydrocarbonized porous silicon
UnTHCPSi+CS	Chitosan-coated undecylenic acid modified thermally hydrocarbonized porous silicon
US FDA	United States Food and Drug Administration
WGA	Wheat germ agglutinin
WHO	World Health Organization

CHAPTER I

Literature review

This chapter was based in the following published or submitted papers/book chapters:

- **Araújo, F.**, das Neves, J., Martins, J.P., Granja, P.L., Santos, H.A. and Sarmento, B. 2016. Functionalized materials for multistage platforms in the oral co-delivery of biologicals. Submitted.
- **Araújo, F.**, Shrestha, N., Granja, P.L., Hirvonen, J.J., Santos, H.A. and Sarmento, B. 2015. Safety and toxicity concerns of orally delivered nanoparticles as drug carriers. Expert Opin Drug Metab Toxicol. 11(3): 381–393.
- **Araújo, F.**, Shrestha, N., Granja, P.L., Hirvonen, J., Santos, H.A. and Sarmento, B. 2014. Antihyperglycemic potential of incretins orally delivered via nano and microsystems and subsequent glucoregulatory effects. Curr Pharm Biotechnol. 15(7): 609-619.
- **Araújo, F.,** Pereira, C., Granja, P.L., Santos, H.A. and Sarmento, B. 2014. Functionalized nanoparticles for targeting the gastrointestinal apical membrane receptors. In *e-book*: Advances and challenges in oral delivery of macromolecules. R. De Vooght-Johnson (Ed.). Future Science Group.
- **Araújo, F**., Fonte, P., Santos, H.A. and Sarmento, B. 2012. Oral delivery of glucagon-like peptide-1 and analogs: alternatives for diabetes control? J Diabetes Sci Technol. 6(6): 1486-97.

1. Diabetes *mellitus*

Diabete *mellitus* (DM) is a group of chronic metabolic diseases, characterized by high blood glucose levels (hyperglycemia) due to disturbances in the metabolism of carbohydrates, fats and proteins (American Diabetes Association, 2009). DM is either caused due to relative or absolute insufficiency in insulin production and/or decreased insulin sensitivity. It is often associated with major complications such as renal failure, retinopathy, amputation, and major risk of myocardial infarction and stroke (Flower, 2008). According to the International Diabetes Federation, in 2015, there were 415 million individuals suffering from DM worldwide, and this number is expected to rise to 642 million by 2040 (International Diabetes Federation, 2016). This makes DM one of the biggest health problems in the world, with very high socio-economic impact. In Europe, around 60 million people suffer from diabetes, out of which 33% remain undiagnosed (International Diabetes Federation, 2016). Portugal, in particular, is estimated to have *ca.* 1 million people suffering from DM (International Diabetes Federation, 2016).

DM can be classified into three main types: Type 1 DM (T1DM), Type 2 DM (T2DM) and gestational diabetes. Other specific types of DM also exist but are not common. T1DM, also known as insulin dependent diabetes *mellitus* or juvenile-onset diabetes, accounts for 5–10% of the total DM cases. T1DM is associated with progressive and specific destruction of pancreatic β cells, caused by cellular-mediated autoimmune destruction, which are responsible for producing insulin, leading to absolute insulin deficiency. T2DM, also known as noninsulin-dependent diabetes *mellitus* or adult-onset diabetes, is a complex disease characterized by resistance to insulin action and inadequate insulin secretion due to pancreatic β-cell dysfunction (Tahrani *et al*., 2010). T2DM accounts for 90–95% of the total DM cases, in which sedentary lifestyle and obesity are often identified as the major causes. According to the World Health Organization (WHO), from 2010−2030, a steep increase of 69% in adults suffering from T2DM is predicted (Shaw *et al*., 2010). Gestational diabetes is, from these three, the least common type of DM. It is associated with pregnancy, without previous diagnosis of DM, which may evolve to T2DM post-pregnancy (American Diabetes Association, 2008; Uma *et al*., 2012; Xu *et al*., 2003).

1.2. Type 2 diabetes *mellitus* (T2DM)

T2DM is not only the most prevalent type of DM, but also one of the most prevalent and rapidly spreading diseases worldwide. The increase in the frequency of occurrence of this disorder and the morbidity associated to the disease leads to a socio-economic burden,

with huge amounts of money being spent every single day (Namba *et al*., 2013; Nicholson and Hall, 2011; Zhang *et al*., 2010). Based on the current understanding of the pathophysiology of T2DM, multiple non-pharmacological interventions (*e.g*., diet and exercise) and pharmacological therapies have been developed to control this disease. However, none of the used therapies have a significant impact on disease progression (Arulmozhi and Portha, 2006; Tahrani *et al*., 2010). Therefore, there is an urgent need to keep on searching for better drugs and delivery systems that could control the disease and reduce the complications and associated side effects.

2. Incretin hormones

Currently, the clinical use of incretins is in the pipeline of T2DM therapy. Incretins are hormones produced by intestinal cells and their function is to enhance the glucose-dependent production of insulin through the target of pancreatic β-cells. The incretin concept relates to the fact that oral food intake provides a more potent insulinotropic stimulus compared to isoglycemic intravenous challenge, reducing postprandial hyperglycaemia (Campbell and Drucker, 2013; Drucker, 2006; Drucker and Nauck, 2006). Thus, incretins are thought to act as amplifiers of the glucose signal, being involved in approximately 50–70% of the total insulin secreted following oral glucose administration (Baggio and Drucker, 2007). As they are glucose-dependent, some side effects of the current therapies, such as hypoglycemia, can be overcome making these hormones the most desirable for therapy (Khan *et al*., 2013; Vilsboll *et al*., 2001). The most important incretin hormones are glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1).

2.1. Glucose-dependent insulinotropic polypeptide (GIP)

GIP was the first described incretin hormone. It is a 42 amino acids peptide secreted by enteroendocrinal K-cells in duodenum and proximal jejunum, which turns into an active form (GIP (1–30)) after being processed by the prohormone convertase 2 (PC2), still in K-cells. It stimulates insulin secretion through its action on the pancreatic β-cells where it up-regulates the biosynthesis and transcription of the insulin gene (Baggio and Drucker, 2007; Campbell and Drucker, 2013).

However, despite all the promising effects of GIP, patients suffering from T2DM were found to be resistant to it, and thus, they have a decrease acute insulinotropic response to native GIP, even with the GIP levels either normal or increased (Bavec, 2014; Tahrani *et al*.,

2010). This resistance is still unclear, but some studies suggest that it may be associated with a down-regulation of GIP receptor expression (Baggio and Drucker, 2007). Moreover, in contrast to GLP-1, described below, it has neither effect on α-cells that secrete glucagon nor in food intake, satiety, and gastric emptying nor body weight (Bavec, 2014; Tahrani *et al*., 2010). Moreover, due to GIP resistance in humans, the majority of clinical studies have focused on the therapeutic potential of GLP-1.

2.2. Glucagon-like peptide-1 (GLP-1)

GLP-1 is an incretin hormone synthetized in enteroendocrinal L cells, at the distal small bowel and colon, as a 37 amino acid peptide (GLP-1 (1–37)) (Campbell and Drucker, 2013). It derives from a proglucagon gene, witch after post-translational processing by prohormone convertase 1/3 (PC 1/3), results into two bioactive circulating peptide forms: GLP-1(7–37) (**Figure 1A**) and GLP-1(7–36) amide (**Figure 1B)**, both with similar potency (Drucker, 2006). In the fasted state, GLP-1 blood values range from 5-10 pmol/L but can reach, with a rapidly increasing, up to 15–50 pmol/L after feeding (Drucker and Nauck, 2006; Nauck, 2011; Nauck *et al*., 2011).

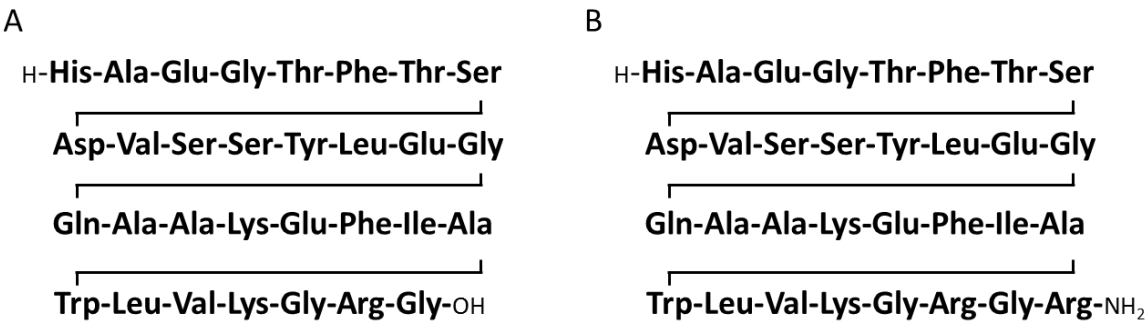

Figure 1. Primary structure of (**A**) GLP-1 (7–37) and (**B**) GLP-1 (7-36) amide.

GLP-1 acts by binding to its G protein-coupled receptor [GLP-1 receptor (GLP-1R)], at membrane of β cells, which activates the GLP-1R signaling pathway (Baggio and Drucker, 2007; Portha *et al*., 2011). As it is not in the scope of this thesis, the molecular mechanisms through which GLP-1 acts will not be addressed, but detailed explanations can be found elsewhere (Baggio and Drucker, 2007; Campbell and Drucker, 2013; Drucker, 2006; Drucker and Nauck, 2006). GLP-1R is widely expressed in the pancreatic islets, stimulating insulin secretion and the neogenesis and proliferation of β cells, thereby decreasing or suppressing the glucagon release (Holst *et al*., 2011; Perfetti and Merkel, 2000). Similarly, GLP-1R are

also expressed along the gastrointestinal tract (Gutzwiller *et al*., 1999), in the brain (Neumiller, 2011) in the heart (Davidson, 2011; Khan *et al*., 2013), and within the liver, kidney, muscle and adipose tissue as can be seen in **Figure 2** (Baggio and Drucker, 2007; Campbell and Drucker, 2013; Drucker, 2006; Drucker and Nauck, 2006).

It is commonly accepted that patients with T2DM have reduced GLP-1 activity due to reduced insulin response to GLP-1. However, despite being controversial, some authors also claim that the secreted GLP-1 is deficient and that is why pancreatic β-cells are not sensitive to it (Nauck *et al.*, 2011). Nevertheless, the insulinotropic effect of GLP-1 remains unchanged, as well as the number of GLP-1R, leading to the restoration of normal GLP-1 activity when external administered in therapeutic doses (Arulmozhi and Portha, 2006; Marre and Penfornis, 2011; Nauck *et al*., 2011; Russell, 2013).

However, GLP-1 has a very short half-life (less than 2 minutes), mainly due to enzymatic degradation caused by dipeptidyl peptidase-4 (DPP-4) enzyme. DPP-4 cleaves GLP-1 in its N-terminal (in the alanine at position 2), resulting in GLP-1(9–36) amide or GLP-1(9–37), both of them inactive peptides (Arulmozhi and Portha, 2006). GLP-1 is further metabolized in the liver which leads to only 2.5–3.75% of the total secreted GLP-1 reaching the systemic circulation (Baggio and Drucker, 2007; Drucker and Nauck, 2006; Holst, 2007).

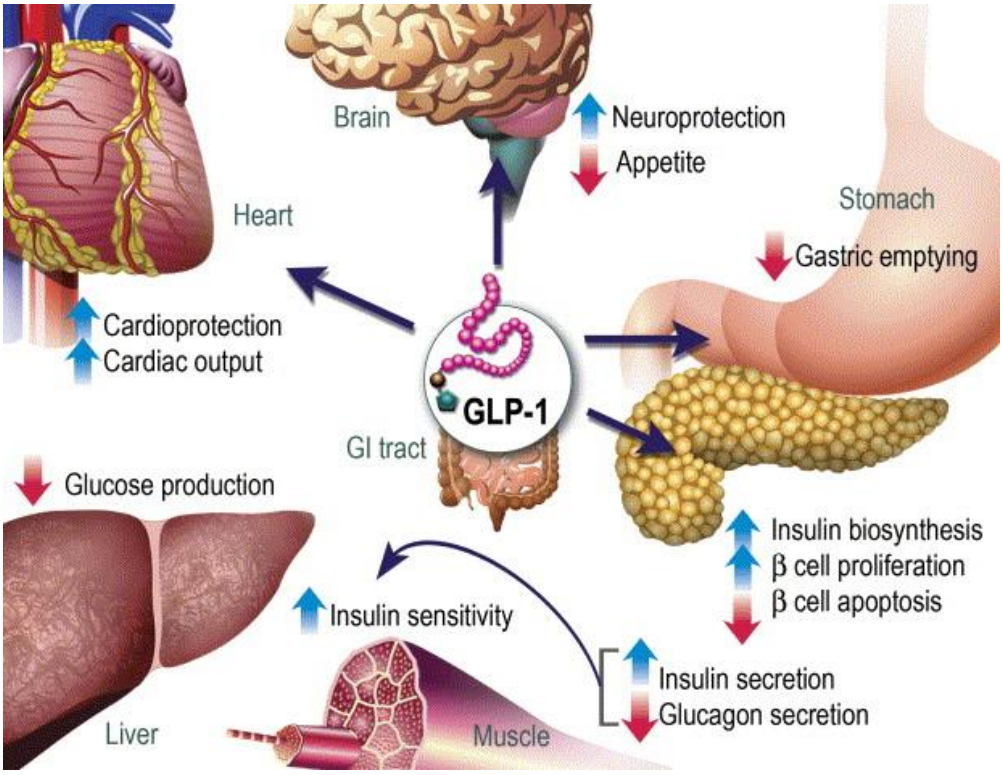

Figure 2. The action of GLP-1 on several peripheral tissues. GLP-1 acts directly on endocrine pancreas, heart, stomach and brain, and indirectly on liver and muscle (Drucker, 2006).

Some strategies to overcome this premature GLP-1 metabolism have been pursued, which resulted in two incretin-based therapies available (using separately or together): the substitution of GLP-1 for GLP-1 analogs and/or the use of DPP-4 inhibitors (Marre and Penfornis, 2011). The pharmacological potency of GLP-1 analogs is much higher than the DPP-4 inhibitors since they are dependent on the glucose levels, on the concentration of endogenous GLP-1 and on the number of the existing receptors. Moreover, DPP-4 besides cleave GLP-1, is also a protease involved in the digestion of Pro/Ala-containing oligopeptides and nutrients and in the absorption of their fragments, a receptor associated with CD45 and adenosine deaminase (ADA), a co-stimulatory for lymphocytes among other functions (Juillerat-Jeanneret, 2013). Thus, the inhibition of DPP-4 actions could also lead to some heath issues (Araújo *et al*., 2012; Gerich, 2013).

GLP-1 analogs used for the treatment of T2DM have been studied for a long time now. GLP-1 analogs are GLP-1 receptor agonists that are structurally similar to native GLP-1, but with some chemical modifications that make them resistant to DPP-4 degradation and delay their clearance from the bloodstream (Unger and Parkin, 2011). This will impart them a longer half-life *in vivo* than native GLP-1. Some of those GLP-1 analogs are in clinical trials and others have been approved by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA), and are already in the market, such as Exenatide (Byetta®) from *Amylin/Eli Lilly* and Liraglutide (Victoza®) from *Novo Nordisk* (**Table 1**) (Li *et al*., 2011). Exenatide is the synthetic form of exendin 4, a peptide isolated from the salivary gland of Gila monster (*Heloderma suspectum*), and presents 53% homology with native GLP-1. In turn, liraglutide is very similar to the native peptide, with 97% homology to GLP-1, differing only in one amino acid and linked with a fatty acid side chain (Araújo *et al*., 2012; Li *et al*., 2011). Both exenatide and liraglutide have been proven to improve the glycemic control (Deacon, 2009; Li *et al*., 2011; Marre and Penfornis, 2011).

Despite alternatives have been developed to improve the problem associated with short half-life of GLP-1, the therapeutic use of GLP-1 analogs is still limited. This is mainly due to the need of parenteral administration, which has a very poor patient adherence, as well as the gastrointestinal side effects encountered in people under this therapy (**Table 1**). The oral route is by far the most desirable way for drug administration, however, the therapeutic activity of labile biomolecules, such as proteins and peptides, is severely compromised when exposed to the harsh physical, chemical and enzymatic gastrointestinal environment due to their physicochemical (solubility), biophysical (conformational and structural stability), and biopharmaceutical (permeability and metabolic stability) properties (Date *et al*., 2016; Pérez *et al*., 2016; Swierczewska *et al*., 2016). In the case of T2DM, it is particularly troublesome due to the chronic therapy nature, requiring frequent drug

Table 1. Characteristics, advantages, disadvantages and stage of drug development (clinical trial/approval) of GLP-1 analogs. Adapted from Khan *et al*. 2013.

Name	Characteristic	Advantages	Disadvantages	Stage of drug development
Exenatide (Byetta) *Amylin/Eli Lilly*	Exendin 4 analogue	Minimal hypoglycemia, counteracts stress-induced hyperglycemia, weight loss	Gastrointestinal side effects	Approved 2005 (USA)
Liraglutide (Victoza) *Novo Nordisk*	GLP-1 analogue	Minimal hypoglycemia, weight loss	Gastrointestinal side effects	Approved 2009 (EU)
Exenatide (Bydureon) *Amylin/Eli Lilly*	Long-acting microencapsulated exendin analogue	Minimal hypoglycemia, weight loss, injected once a week	Gastrointestinal side effects	Approved 2011 (EU)
Lixisenatide (Lyxumia) *Sanofi*	Exendin 4 analogue	Minimal hypoglycemia, superior reduction in postprandial plasma glucose, weight loss	Gastrointestinal side effects	Approved 2013 (EU)
Albiglutide *GlaxoSmithKline*	Long-acting GLP-1 analogue	Minimal hypoglycemia, improved cardiovascular outcomes, weight loss, injected once a week or month	Minor gastrointestinal side effects	Approved 2014 (EU and USA)
Taspoglutide *Roche/Ipsen*	Long-acting GLP-1 analogue	Minimal hypoglycemia, weight loss, injected once a week	Very severe gastrointestinal side effects	Phase III clinical trials
Dulaglutide *Eli Lilly*	Long-acting GLP-1 analogue	Minimal hypoglycemia, weight loss, reduced immunogenicity and renal clearance, injected once a week	Gastrointestinal side effects	Approved 2014 (USA)
CJC-1134-PC *ConjuChem*	Long-acting exendin 4 analogue	Minimal hypoglycemia, reduced renal clearance, weight loss, injected once a week	Minor gastrointestinal side effects	Phase II clinical trials
Semaglutide *Novo Nordisk*	Long-acting GLP-1 analogue	Half-life of 160 h. Reduced risks of cardiovascular disease	Mild-moderate gastrointestinal side effects	Phase III clinical trials
Deglutec and liraglutide (IdegLira) *Novo Nordisk*	Long-acting insulin analogue and GLP-1 analogue combination	Weight loss, effective against elevated fasting and postprandial plasma glucose	Hypoglycemia. Minor gastrointestinal side effects	Approved 2015 (EU and USA)

administrations, in some cases several times daily, which leads to poor patient compliance (Araújo *et al*., 2015a; Gupta *et al*., 2013).

3. Barriers to the oral delivery of biologicals

The gastrointestinal tract provides an optimum environment for the digestion of ingested macromolecules from diet into their molecular building blocks, which can then be easily absorbed in the intestine (Chen *et al*., 2011). Besides contributing to basic nutritional intake, the digestion process accomplished by enzymes and pH variations found along the gastrointestinal tract, as well as the gut mobility, also acts as a gatekeeper of the human body, being the first line of defense against pathogens and xenobiotics (Araújo *et al*., 2012). Likewise, the natural course of the orally administrated therapeutic biomolecules will lead to their entrapment within the gastrointestinal lumen and eventual breakdown, with consequent structure and bioactivity loss (Chen *et al*., 2011). Even if the biochemical barriers can be overcome, when reaching the absorptive interfaces, therapeutic biomolecules would face other frequently unsurpassable hurdles, such as the mucus layer and the epithelial cell lining that limit their permeation/absorption and efficient translocation to the blood circulation (**Figure 2**). Understanding the characteristics and functions of these three main barriers is crucial in designing and developing effective carriers for the oral administration of therapeutic biomolecules.

3.1. The biochemical barrier

The biochemical is the first barrier that peptides and proteins face after oral administration. It essentially comprises different pH values and the enzymatic activity that occur within the gastrointestinal tract. Hydrogen ion concentrations oscillate widely, with very acidic values (pH 1.2−3) at the stomach, but dramatically increasing to slightly alkaline in the intestine (pH 6.5−8.0) in the fasted state (**Figure 3**) (Yun *et al*., 2013). Peptides and proteins are susceptible to these changes, which may lead to their oxidation, deamination or hydrolysis, thus potentially causing loss of activity (Yun *et al*., 2013). They are also vulnerable to the enzymatic activity that drives their degradation (Rekha and Sharma, 2013). These enzymes can be found in the luminal space of the gastrointestinal tract, namely pepsin (at gastric level) or trypsin (in the intestine), being the greatest enzymatic activity observed at the brush border in the cytosol and in the cellular organelles (*e.g.*, lysosomes) of the enterocytic intestinal cells of the duodenum and jejunum. Due to their immune nature,

Peyer's patches of the jejunum and the ileum also present high catabolic activity, with around 20−30% of the total intestinal enzymatic activity (Rekha and Sharma, 2013; Roger *et al*., 2010; Yun *et al*., 2013).

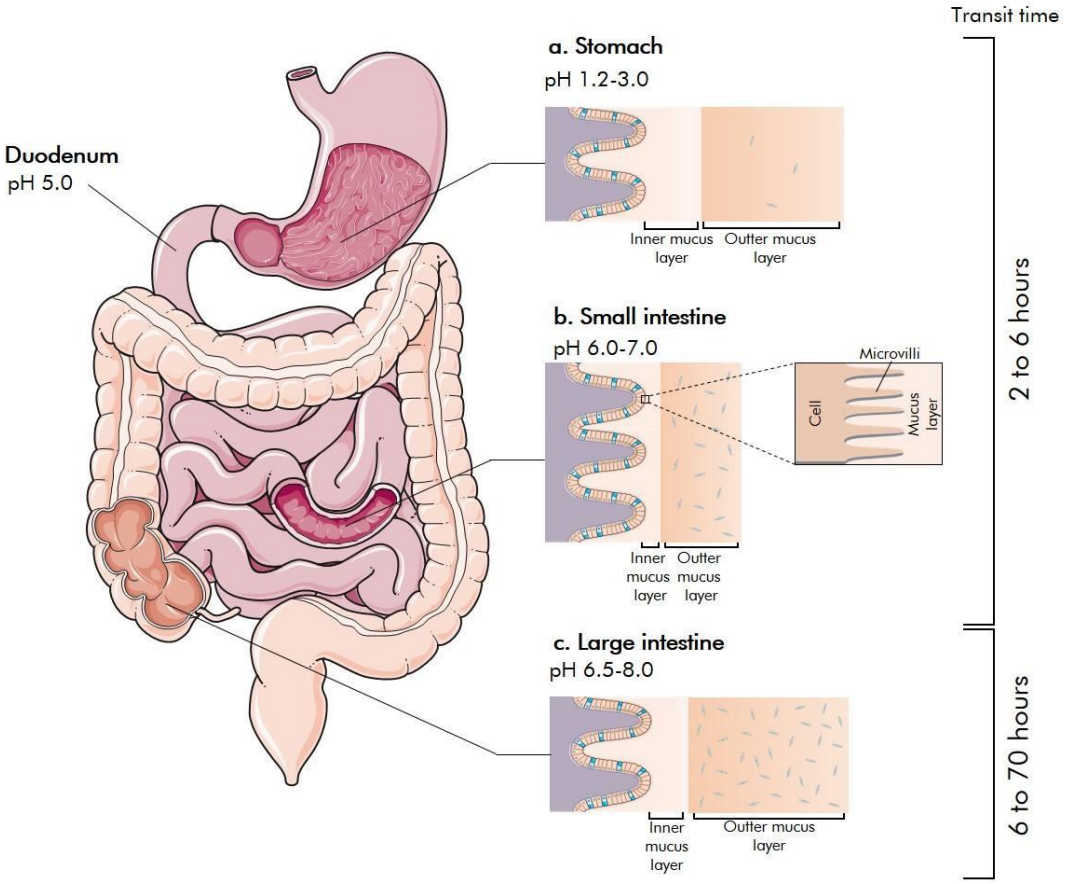

Figure 3. Gastrointestinal tract barriers to the oral delivery of biomolecules. Figure produced using Servier Medical Art.

3.2. The mucus barrier

The mucus layer is another challenging obstacle to be overcome. In fact, this is the first physical barrier encountered by peptides and proteins after their oral administration (Cone, 2009; Ensign *et al*., 2012b). Mostly constituted by water, mucin glycoproteins (1-5%) and electrolytes, mucus comprises a randomly organized mucin fiber mesh, forming a heterogeneous 3D scaffold-like structure of variable porosity (Johansson *et al*., 2011). Besides molecular entanglement, binding between these fibers occurs mostly by disulfide linkages and hydrophobic interactions that form dense mesh-pores (50–1800 nm) creating a steric and potentially highly adhesive barrier to the transport of biomolecules and supra-

molecular structures, including drug delivery systems, from the intestinal lumen to the mucosal tissue (Lai *et al*., 2009; Sosnik *et al*., 2014; Thornton and Sheehan, 2004). In general, structures above 1000 nm are unable to permeate the mucus due to the limited pore size of the mucin mesh (das Neves *et al*., 2011). Mucins are rich in both negatively-charged glycosylated regions and hydrophobic domains, making it possible the establishment of interactions of variable nature (Ensign *et al*., 2012b). The typical viscoelastic properties of mucus layer allow for this sticky layer to protect the gastrointestinal epithelial cell lining from mechanical damage. Mucus also acts as a buffer barrier to pH oscillations in the gastrointestinal lumen. It rapidly traps and removes large molecules and matter due to fast turnover and clearance, usually measured from minutes to few hours (Lai *et al*., 2009; Tang *et al*., 2009). Due to its important protection role, mucus is continuously secreted and spread throughout the entire gastrointestinal tract, with the help of the peristaltic movements. Mucus blanket is determined by the balance between the rate of secretion and the rate of degradation and shedding, and in a balance between the protective capability and nutrient absorption rate (Atuma *et al*., 2001; Cone, 2009). According to the different locations, mucus presents different thickness: it is thicker at the stomach and colon, which helps protecting the epithelial cell lining from the acidic pH and bacteria, respectively, while in the small intestine, the major region of nutrients absorption, it is thinner and more loosely adherent to the mucosa, varying greatly on digestive activity (Ensign *et al*., 2012b; Rekha and Sharma, 2013; Yun *et al*., 2013). Penetration and transport across this mucus barrier is necessary in order to reach the absorptive epithelial cells. Interested readers can find excellent reviews on the mucus barrier to macromolecules and nanoparticles published over the past few years (Cone, 2009; Ensign *et al*., 2012b; Lai *et al*., 2009).

3.3. The epithelial barrier

As previously mentioned, the harsh acidic environment and the presence of enzymes, as well as its small surface area, reduce the stomach to an unfeasible site for biomolecules absorption (Renukuntla *et al*., 2013). In contrast, the human intestinal epithelium is a highly absorptive region. With a tightly arranged structure, the small intestine is made-up of a monolayer of polarized epithelial cells, organized into crypts and villi. The small intestinal epithelium is mainly composed by enterocytes, which present at their apical side well-ordered projections called microvilli greatly increase the absorptive area, rendering a total intestinal adult surface area of 300–400 m^2 (Date *et al*., 2016; Pawar *et al*., 2014). Besides the enterocytes, the intestine also comprises mucus-secreting goblet cells, the second most abundant cell type, interspersed between the remaining intestinal cells. Antimicrobial

peptides-secreting Paneth cells are also present in the crypts, as well as the hormone-secreting enteroendocrine cells (Gerbe *et al*., 2012; Pridgen *et al*., 2015). Another cell type, the specialized microfold (M) cells, are responsible for antigen transportation through a non-degradative pathway to dendritic cells, and thus, may constitute a good target to the delivery/absorption of biomolecules (Kristensen and Nielsen, 2016; Pawar *et al*., 2014).

While the small intestine is primarily responsible for digestion and absorption of nutrients, the colon participates in the maintenance of fluid and electrolyte balance, not being ideally suited for promoting the absorption of biomolecules. Moreover, colon does not fold upon itself to create additional microvilli, limiting the available surface area (Pawar *et al*., 2014). However, colon may offer some advantages over the small intestine, such as lower levels of enzymatic activity, higher responsiveness to permeation enhancers, and prolonged transit/residence time (Maroni *et al*., 2012).

The intestinal epithelium rests on top of the basal lamina (extracellular matrix), which separates it from the lamina propria. After being transported across the epithelial lining, molecules transpose into the lamina propria, which contains a network of capillaries that is responsible for their drainage into blood circulation. The capillaries converge into venules and, eventually, the portal vein that conveys material to the liver (Pridgen *et al*., 2015). All of the epithelial cells are interconnected with each other by tight junctions that have paramount importance in retaining the polarization of the epithelial cells and maintaining the integrity of the epithelium (Tsukita *et al*., 2001) (**Figure 4**). Tight junctions are structures formed by several proteins, including transmembrane proteins (occludin and claudin), cytoplasmic plaque proteins (*Zona Occludens* 1, 2 and 3 (ZO-1, ZO-2, ZO-3), cingulin, and 7H6) and regulatory proteins (Salama *et al*., 2006). This yields to very narrow spaces between neighboring cells, with typically average gap sizes of less than 15 Å (Pawar *et al*., 2014). The assemble of these junctional proteins is dynamic and they are constantly remodeling, forming pores possessing size and charge selectivity, which act as strict regulators of permeation through the paracellular space (Anderson and Van Itallie, 2009; Van Itallie *et al*., 2008). However, the paracellular space comprises less than 1% of the intestinal surface area, thus limiting the transport through this route to small hydrophilic drugs up to 700 Da (smaller than 1 nm) (Chen *et al*., 2011; Renukuntla *et al*., 2013).

On the other hand, transport across cells (transcellular transport) is largely limited by the cell membranes, consisting mainly of phospholipid bilayers, and restricted to lipophilic molecules (Chen *et al*., 2011). However, cells are able to use other mechanisms then just the passive diffusion and can take-up biomolecules with hydrophilic nature. This transport pathway may be divided into different endocytic mechanisms: phagocytosis, macropinocytosis, clathrin-mediated endocytosis and caveole-mediated endocytosis, which are adenosine triphosphate (ATP) dependents, and clathrin- and caveolae-independent

endocytosis, which are not ATP dependents (Chen *et al*., 2011; Conner and Schmid, 2003). These uptake mechanisms occur at the apical cell membrane, where biomolecules are taken-up, and then the transport occurs through cells within vesicles, releasing their contents at the basolateral pole (Chen *et al*., 2011). Thus, the transcellular transport is the most probable route for the absorption of biomolecules.

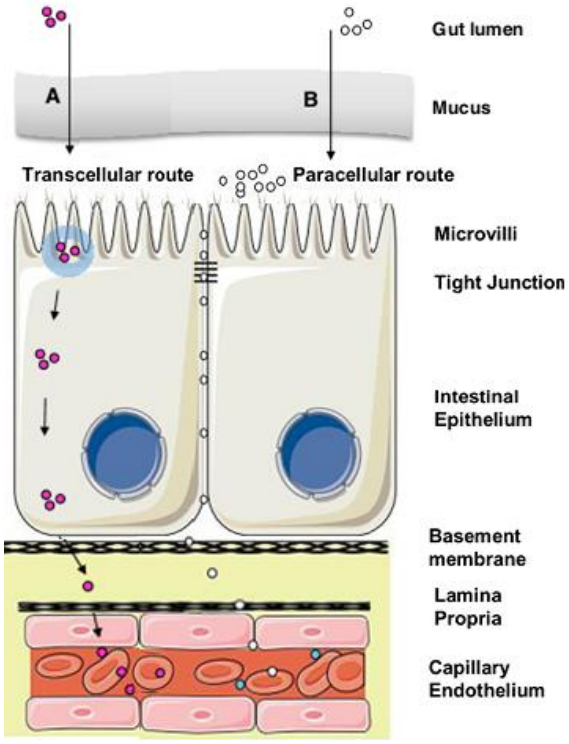

Figure 4. The physical barrier of the intestinal epithelium to passage of peptides and proteins from the gut lumen to the basal lamina propria. Molecules may cross the cell membrane barrier by different mechanisms: (**A**) transcellular transport or (**B**) paracellular transport. Adapted from (Aguirre *et al*., 2016).

4. Multistage platforms based in particulate carrier systems for the oral delivery of peptides and proteins

More than ever, the field of materials science is providing interesting tools to meet the challenge of developing drug carriers that are able to orally deliver peptides and proteins in a controlled manner and overcome the gastrointestinal hurdles, enhancing their bioavailability (Nitta and Numata, 2013). Polymeric, porous and lipid-based particulate delivery systems are the most commonly used for this purpose. The main advantages of these systems are the

protection of the peptides and proteins from degradation, the ability to control nanoparticle physicochemical properties, such as size and charge, and the high surface-volume ratio (Danhier *et al*., 2012; Yun *et al*., 2013; des Rieux *et al*., 2006).

Among the polymers, poly(lactic-co-glycolic acid) (PLGA), an aliphatic synthetic polyester co-polymer, is one of the most used to produce nanoparticles for the oral delivery of peptides and proteins. Besides the advantages above referred, PLGA is biodegradable and biocompatible, being FDA and EMA approved in drug delivery systems for parenteral administration. PLGA features can be modulated by the ratio between the monomers which constitute the polymer, exhibiting a wide range of erosion times, favorable degradation characteristics, leading to sustained release profiles, and tunable mechanical properties. There is also the possibility to modify the surface properties of PLGA particles and the described formulations and methods of production can be adapted to various types of drugs (Araújo *et al*., 2014; Fonte *et al*., 2015). Moreover there are several *in vitro* and *in vivo* studies demonstrating its efficiency (Araújo *et al*., 2014; Danhier *et al*., 2012; Reix *et al*., 2012; Santander *et al*., 2009). However, poor drug loading (≈ 1%) and possible drug degradation, due to exposure to organic solvents and high shear stress during the preparation process, are disadvantages associated with their production techniques (Danhier *et al*., 2012).

Lipid-based carriers have well-known safety profiles, with some of them already established for large-scale production (Muller *et al*., 2011). As compared to some other systems, the solid-lipid nanoparticles (SLN), which represent a class of colloidal particles composed of lipids that are solid at both room and body temperatures, are promising. They have additional benefits to protect the encapsulated peptides and proteins and their physical stability, enhanced pharmacokinetic properties and modulated release of drugs (Geszke-Moritz and Moritz, 2016). However, the SLN are often associated with poor drug encapsulation and loading, and drug release after polymorphic transition during storage. Moreover, the conditions used for their production can be severe, with the use of organic solvents and high temperatures (Geszke-Moritz and Moritz, 2016; Mehnert and Mader, 2001).

Between the porous-biomaterials, porous silicon (PSi) has attracted a lot of attention for the administrations of proteins and peptides (Santos *et al*., 2014). PSi-based particulate systems have a large surface area and pore volume with adjustable diameters (2 - 50 nm), that can be easily controlled by altering the fabrication parameters, as well as higher drug loading capacity compared to the majority of the other materials. They present high stability, biocompatibility and biodegradability (Liu *et al*., 2013a; Liu *et al*., 2014; Salonen and Lehto, 2008; Santos *et al*., 2014; Shahbazi *et al*., 2013). The modification of their surface is an easy process and it offers a unique advantage of mild aqueous drug loading conditions and the

possibility to avoid the use of harsh organic solvents, making it a simple process where the drug is retained inside the pores by physical adsorption or electrostatic interactions (Salonen and Lehto, 2008; Santos *et al*., 2014; Shahbazi *et al*., 2014). The top-down fabrication process also allows an easy scaling-up (Mäkilä *et al*., 2014). Likewise other particular systems, also PSi systems have some drawbacks. To produce the particles, very time consuming techniques need to be employed wich leads to a more expensive material in comparison to the other materials. Besides that, they have a burst release of loaded proteins and peptides and their safety profile is not established yet (Araújo *et al*., 2014; Shahbazi *et al*., 2014; Shrestha *et al*., 2015).

Several strategies based on these three particulate delivery systems (polymeric-based (Attivi *et al*., 2005; Damge *et al*., 2007; Ensign *et al*., 2012a), lipid-based (Li *et al*., 2013; Zhang *et al*., 2006; Zhang *et al*., 2009; Zhang *et al*., 2012) and porous-based particulate delivery systems (Huotari *et al*., 2013; Kilpelainen *et al*., 2011)) have been proposed over the last years to address the abovementioned concerns for the oral administration of GLP-1 and GLP-1 analogs. However, none of the systems used for the oral administration of GLP-1 and GLP-1 analogs was able to fully address all the needs to overcome the barriers of the gastrointestinal milieu. Indeed, despite all the investment and time spent on the development of particulate systems over the past 40 years, not only for GLP-1 but also for other biopharmaceuticals as well, few formulations have reached clinical testing and significantly impacted the human healthcare (Valencia *et al*., 2013).

Thus, continuous efforts to understand these complex biomaterial−biological interactions would lead to ameliorate surface functionalities of particulate drug delivery systems. The functionalization of biologically inactive and biocompatible materials, to impart precise biological functions and provide physicochemical characteristics that can radically change the properties observed in bulk materials is considered a promising approach in drug delivery (Goldberg *et al*., 2007; Solanki *et al*., 2008).

The major advantages of the drug delivery system functionalization are: (i) the improvement of retention and transport properties; (ii) the increase in drug solubility, diffusivity and distribution along mucosal fluids; and (iii) the potential for targeted drug delivery to a specific tissue with minimal distribution to the surrounding ones (Lopes *et al*., 2016; Plapied *et al*., 2011). Some important aspects of particle surface functionalization, however, need to be addressed during translation from experimental success to clinical practice (Mout *et al*., 2012). These are summarized in **Table 2** and discussed in detailed elsewhere (Araújo *et al*., 2016b).

Table 2. Summary of the most important parameters and respective considerations for an adequate particle surface functionalization.

Parameter	Observations	Refs.
Conjugation methods	Covalent conjugation is stronger and long lasting; Non-covalent conjugation is simpler and occurs spontaneously.	(Doane and Burda, 2013; Leitner *et al*., 2003)
Hydrodynamic size	Interaction between particles-cells is highly dependent on particle size: smaller particles present higher interaction when compared to larger particles.	(Byrne *et al*., 2008; Gaumet *et al*., 2008; Nel *et al*., 2009; Roger *et al*., 2010; Shinde Patil *et al*., 2001)
Surface charge	Particularly important for non-covalent conjugation methods; Strong surface charge is often advocated to guarantee colloidal stability of the particulate systems; Positively charged particles exhibit higher internalization into cells; Neutrally charged particles avoid non-specific interactions *in vivo.*	(Bourganis *et al*., 2015; Cho *et al*., 2009; das Neves *et al*., 2012; Griffin *et al*., 2016; Hirsch *et al*., 2013; Kaaki *et al*., 2012; Moulari *et al*., 2013; Plapied *et al*., 2011; Rahme *et al*., 2013; Verma and Stellacci, 2010; Yu *et al*., 2011)
System shape and surface topography	Affect particles diffusivity and adhesion to cells; Spherical particles are internalized more efficiently due to constant contact angle with cells than other shaped particles; Interactions with cells are enhanced when particles present a rough topography.	(Agueros *et al*., 2009; Amin *et al*., 2015; Champion *et al*., 2007; des Rieux *et al*., 2013; Dunne *et al*., 2000; Nel *et al*., 2009; Niu *et al*., 2013; Panyam *et al*., 2003)
Timing of conjugation	Before production of the particles: conditions in which surface functionalization takes place may promote the release of active molecules and cross-reaction may occur with biopharmaceuticals; After production of the particles: poor control over the extent of ligand coupling to the surface of the particles after production.	(des Rieux *et al*., 2013; Plapied *et al*., 2011)

Nevertheless, to successfully fulfill the oral delivery of peptides and proteins, there is the need to evolve and take advantage of the enormous progress made in biomaterials science, to design "smart" particulate drug delivery systems that can respond to the gastrointestinal environmental features. These characteristics may be variations in pH, ionic strength, redox potential or enzymatic activity (Gao *et al*., 2010). The response to stimuli can assume many forms like changing size, shape, surface characteristics, biomaterial solubility, degree of intermolecular association with the cargo, among others (You *et al*., 2010). Similarly, particles can also be tuned to provide selective or specific recognition and/or biocompatibility (De *et al*., 2009).

However, in certain cases, the use of individual materials and/or the manufacturing approaches to produce the particulate systems seem to compromise the potential of the final carrier, failing the goals to what they were designed for. To overcome these limitations, several techniques are being employed for adequate tailoring of drug delivery systems, and achieve what are called multistage platforms. A multistage platform can comprise one or more biomaterials, as individual or composite, resulting in entirely new systems embracing the best properties of each material. Biomaterials with tailored surface design are demonstrating to be exciting multifunctional tools that hold promise in overcoming the failures of the delivery of biopharmaceuticals based on traditional drug dosage forms and drug delivery approaches. Interestingly, such platforms may even capitalize from otherwise potentially deleterious features of the gastrointestinal tract to improve the absorption of orally delivered biopharmaceuticals. A summary of the main strategies and respective platforms can be found in **Table 3**.

Table 3. Strategies to improve absorption of biopharmaceutics loaded into particulate-based oral delivery systems.

Parameter	Strategies	Platforms	Refs.
Mucoadhesion	Increasing the gastrointestinal retention time of the carrier systems and drugs	CS coated liposomes Mixed micelles PAA-coated nanoparticles Pectin-liposome nanocomplexes PEG coated nanoparticles pHPMA coated nanoparticles PMVC microparticles Polydimethylaminoethylmethacrylate sub-microparticles SLNs TMC nanoparticles	(Bajka *et al*., 2015; Coco *et al*., 2013; Cui *et al*., 2006; Fricker *et al*., 2010; Han *et al*., 2012; Kollner *et al*., 2015; Muller *et al*., 2014; Netsomboon and Bernkop-Schnurch, 2016; Niebel *et al*., 2012; Perera *et al*., 2015; Sajeesh and Sharma, 2011; Shan *et al*., 2015; Sonia and Sharma, 2013; Thirawong *et al*., 2008; Tobio *et al*., 2000; Wilcox *et al*., 2015)
pH	pH-Sensitive platforms for tailoring the release of biopharmaceuticals	BLG-pectin composite CS coated PLGA nanoparticles CS-Eudragit® composite CS nanoparticles PLGA-Eudragit® composite PLGA/porous silicon nanoparticles encapsulated in HPMC-AS polymer	(Abouelmagd *et al*., 2015; Araújo *et al*., 2015b; Izadi *et al*., 2016; Jelvehgari *et al*., 2010; Makhlof *et al*., 2009; Mukhopadhyay *et al*., 2015; Prasad and Dangi, 2015; Sharma *et al*., 2011; Shrestha *et al*., 2015; Sonaje *et al*., 2010a; Sun *et al*., 2015; Wang *et al*., 2015)

Active and passive targeting	Targeting at the absorption site: membrane transporters and receptors on the gastrointestinal tract	Fc fragment conjugated to PLA-PEG nanoparticles Fab' portion of the F4/80 Ab conjugated to PLGA-PEG nanoparticles UEA-1 modified PLGA nanoparticles and liposomes Alginate coated CS nanoparticles WGA functionalized PLGA nanoparticles Hydrophobic NPs (PS, PMMA, PHB, PLA, PLGA) AAL targeted albumin microparticle	(Gao *et al*., 2013; Harokopakis *et al*., 1998; Jin *et al*., 2012; Kang *et al*., 2008; Laroui *et al*., 2014; Pridgen *et al*., 2013; Rajapaksa *et al*., 2010; Roger *et al*., 2012; Wang *et al*., 2010; Xiao *et al*., 2013; Xiao *et al*., 2014)
Redox potential	Redox potential-responsive drug carriers for controlled release	Thioketal nanoparticles Glutathione treated single-protein nanocapsules CS and PASP composite RNP^{O}	(Vong *et al*., 2012; Wilson *et al*., 2010; Zhao *et al*., 2011; Zheng *et al*., 2013)
Absorption enhancers and enzymatic inhibitors	Co-delivery of biopharmaceuticals with other modulators	CS-PLGA nanoparticles conjugated with CPP (R8) CS-porous silicon nanoparticles conjugated with CPP (R8) CS-PLGA nanoparticles conjugated with CPP (Tat) SNEDDS conjugated with CPP (Tat) CS-PLGA nanoparticles conjugated with CPP (R8) encapsulated in HPMC-AS loaded with iDPP4 CS-porous silicon nanoparticles coated with HPMC-AS loaded with iDPP4 Na-CMC covalently bound to elastinal and BBI	(Araújo *et al*., 2016; Araújo *et al*., 2015b; Guo *et al*., 2016; Mahmood *et al*., 2016; Marschutz and Bernkop-Schnurch, 2000; Shrestha *et al*., 2016; Shrestha *et al*., 2015; Werle *et al*., 2007)

Abbreviations: AAL. Aleuria aurantia lectin; **BBI.** Bowman-Birk inhibitor; **BLG.** β-lactoglobulin; **CS.** Chitosan; **CPP.** Cell Penetrating Peptide; **HPMC-AS.** Hydroxypropyl methyl cellulose acetate succinate; **IBD.** Inflammatory bowel disease; **iDPP4 –** DPP4 inhibitor; **LMW.** Low molecular weight; **Na-CMC.** Sodium carboxymethylcellulose; **PAA.** Poly(acrylic acid); **PASP.** Poly($_L$-aspartic acid); **PEG.** Poly(ethylene glycol); **PHB.** Poly(hydroxybutyrate); **pHPMA.** N-(2-hydroxypropyl) methacrylamide copolymer; **PLGA.** Poly(lactide-co-glycolide); **PMMA.** Poly(methyl methacrylate); **PMVC.** Poly (methacrylic acid-vinyl pyrrolidone)-chitosan; **PPM.** Mannosylated bioreducible cationic polymer; **PS.** Polystyrene; **RNP^O.** Nitroxide radical-containing nanoparticle; **SNEDDS.** Self-nanoemulsifying drug delivery systems; **SLN.** Solid lipid nanoparticle; **TMC.** Trimethyl chitosan; **UEA-1.** Ulex europaeus 1 lectin; **WGA.** Wheat germ agglutinin.

4.1. Increasing the gastrointestinal retention time

In order to avoid short transit time through the entire gastrointestinal tract, and thus, potentially increase the absorption of biopharmaceuticals, several drug delivery systems have been surface functionalized towards higher association with the mucus. This may allow slowing particle transport to the time scale of mucus renewal, avoiding their early clearance and increasing the proximity of peptides and proteins with the absorption site. Hydrophilic polymers possessing polar functional groups capable of forming hydrogen bonds with mucosal surfaces typically have excellent mucoadhesive properties (Andrews *et al*., 2009; Khutoryanskiy, 2011). The higher the density of surface functional groups able to establish adhesive bounds, the stronger the interaction with mucus will be (Andrews *et al*., 2009; Khutoryanskiy, 2011). Mucoadhesive polymers should have appropriate features regarding their concentration, flexibility, molecular weight, conformation, surface tension, chain structure and length, which are detailed elsewhere (Andrews *et al*., 2009; Rekha and Sharma, 2013). Among other commonly used polymers (described in more detail by Sosnik *et al*. (Sosnik *et al*., 2014)), such as alginate and derivatives (Deat-Laine *et al*., 2013; Yang *et al*., 2011a), pectin (Gungor *et al*., 2010; Li *et al*., 2014; Sharma *et al*., 2012), poly(acrylic acid) (PAA) (Kriwet *et al*., 1998; Makhlof *et al*., 2011) and cellulose derivatives (Rahmat *et al*., 2013; Suwannateep *et al*., 2011), CS is one of the most well-investigated and most used (Andrade *et al*., 2011; Prego *et al*., 2005; Takeuchi *et al*., 2001), featuring excellent ability to interact with mucus (Sigurdsson *et al*., 2006; Sosnik *et al*., 2014).

Particles functionalized with mucoadhesive polymers are able to bind to mucins at mucosae by either specific or non-specific interactions. Bonding may be categorized into ionic, covalent, hydrogen and van der Waals' forces and hydrophobic interactions (Smart, 2005). Despite several decades of research, the mucoadhesion phenomenon is still not fully understood. There are several theories that attempt to explain mucoadhesion mechanisms (Sosnik *et al*., 2014). However, due to the complex nature of adhesive interactions, these theories can only provide satisfactory understanding of mucoadhesion when combined. The more consensual theory in the biomedical field though is the diffusion (or interpenetration) theory. Briefly, it considers that after intimate contact is established, polymer chains diffuse into and entangle with the mucin fibers composing mucus, while simultaneously establishing adhesive bonding. The consolidation of adhesion occurs through the dynamic balance between diffusion, physical entanglement and adhesive/repulsive interactions (Rekha and Sharma, 2013; Smart, 2005).

Considering all the characteristics summarized in **Table 2**, such as the size and charge of the delivery systems, maximum mucoadhesive potential can only be achieved if multiple features of particulate systems are considered. Owing to the high surface area-to-

volume ratio, nanoparticulate systems are once more promising carriers for the oral delivery of peptides and proteins, since the interface available to establish bonding with mucus dramatically increases, thus promoting long lasting bonding (Prego *et al*., 2005). Theoretically, decreasing size is associated with more intimate interactions and prolonging of residence time and drug oral bioavailability. Due to the width of the mesh spaces delimited by mucin fibers, it is expected that particulate systems below 1000 nm do not only adhere to the mucus, but also potentially penetrate it without causing harm (das Neves *et al*., 2011; Pearson *et al*., 2016). It was recently demonstrated by Bajka *et al*. that 100 nm bile salt coated particles diffuse more rapidly through porcine intestinal mucus than 500 nm bile salt coated particles (Bajka *et al*., 2015). More important than size, surface properties are able to define the adhesive behavior of nanosystems within the range of about 100−500 nm (Tang *et al*., 2009; Yang *et al*., 2011b). Indeed, as long as adhesive interactions are minimal, larger particles can also diffuse through mucus. Thirawonget *et al*. produced liposomes conjugated with pectin to improve the oral bioavailability of calcitonin (Thirawong *et al*., 2008). Modified liposomes were larger but had prolonged residence time in the gut, over at least 6 h, as compared to plain liposomes (Thirawong *et al*., 2008).

One additional interesting approach might be the sequential combination of both adhesive and penetrating properties in the same system. In one hand, mucoadhesion may contribute for prolonged residence time close to the absorption site while, on the other hand, overcoming the mucus barrier may enhance diffusion towards the absorptive surfaces of the gut. Köllner *et al.* presented a study in which they tested different drug delivery systems, namely a mucoadhesive nanocarrier based on thiolated PAA nanoparticles (PAA-cys), papain-modified PAA (PAA-pap) nanoparticles exhibiting mucolytic properties to enhance particle diffusion into deeper mucus regions before adhesion, as well as particles containing both conjugates (PAA-cys-pap nanoparticles) (Kollner *et al*., 2015). All the systems had a size range between 158−214 nm and presented negative zeta potential values. Reported data showed that the combination of both conjugates had a 2.0-fold higher penetration into the mucus layer in comparison with PAA-cys nanoparticles and a 1.9-fold increase in mucoadhesion for the nanoparticulate system based on thiolated PAA compared to PAA-pap nanoparticles (rheological studies). This render to the particles a higher retention time at the absorption site as well as a more intimacy contact with the epithelial cells (Kollner *et al*., 2015).

As mentioned before, the effect of size is strongly dependent on the particle surface chemistry, in particular surface charge (Yildiz *et al*., 2015). Mucins have a low isoelectric point, which determines its negative charge at all physiological pH values of the gastrointestinal tract. This severely affects its interaction with the carrier systems as particles will undergo either repulsive or attractive electrostatic forces depending on whether these

systems present negative or positive surface charge, respectively. Thus, positively charged particles tend to present higher mucoadhesiveness even though there are some other players than just the electrostatic interactions that do not follow this “rule”, as for thyomers (Fonte *et al*., 2011; Miller *et al*., 1998; Shrestha *et al*., 2015). By contrast, negatively charged and uncharged particles, while still being able to promote hydrophobic bonding and van der Waals’ forces with mucin, are prone to have more mucopenetrating capacity (Netsomboon and Bernkop-Schnurch, 2016). The functionalization of nanoparticulate systems with polyethylene glycol (PEG) is the most commonly used strategy to render the systems mucopenetrating properties (Moulari *et al*., 2013). PEG not only renders a more neutral charge to the systems, but also make these more hydrophilic promoting unhindered transport across the mucus, which facilitates reaching intestinal epithelial cells and has the potential to enhance bioavailability (Crater and Carrier, 2010; Maisel *et al*., 2015; Plapied *et al*., 2011; Xu *et al*., 2015).

Using a system able of changing its surface charge from slightly negative to positive values at the intestinal level, for example, under the influence of intestinal alkaline phosphatase, may be an interesting approach to enhance the delivery of biopharmaceuticals (**Figure 5**) (Bonengel *et al*., 2015; Netsomboon and Bernkop-Schnurch, 2016; Perera *et al*., 2015). Such carriers are able to feature unhindered transport until the intestine without significantly binding to gastric mucus or mucosa, but dramatically change their behavior to mucoadhesive by enzymatic influence. This possibility may favor the drug release at the absorptive site, *i.e.*, the intestine, while abbreviating gastric residence and consequent loss of active biopharmaceuticals.

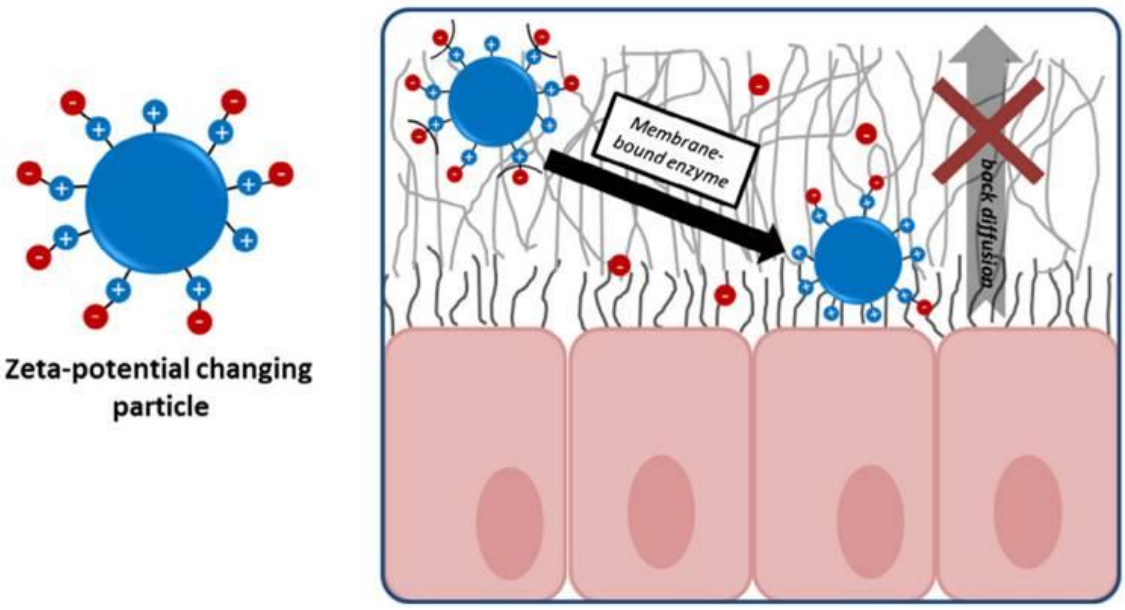

Figure 5. Schematic represents the design of a system capable of changing its zeta-potential from slightly negative to positive values at the intestinal level, favoring drug release at the absorptive site (Pereira de Sousa *et al*., 2015).

Besides charge, specific chemical moieties that may be present at the surface of particulate systems can also impact on the mucoadhesive behavior. The immobilization of mucolytic enzymes, such as papain, on the surface of nanoparticles will render them a mucopenetrating permeability of functionalized than plain particles. When formulated as enteric coated capsules and orally administered in Sprague Dawley rats, Muller *et al*. detected that the majority of papain-functionalized nanoparticles were able to traverse across the mucus layer and remained in the duodenum and jejunum of the small intestine, where drug absorption primarily occurs (Muller *et al*., 2014). A similar study was conducted by Pereira de Sousa *et al.* where they show that besides papain also bromelain can be used as a mucopenetrating agent character (Pereira de Sousa *et al*., 2015). The viscosity of the mucus strongly decreases leading to 3-fold higher penetration.

Overall, particulate systems with mucoadhesive properties are able to prolong the residence time in the gastrointestinal tract, ameliorating the contact between carrier systems and the mucus that covers the epithelial cells. In turn, mucopenetrating capacity has gained a lot of attention in the last years, since it allows a closer contact between the particles and the cells, thus increasing intestinal permeability. There is still a lot to discuss and to investigate regarding if one characteristic is better than the other, but a dual behavior of the particulate systems can be advantageous over a single one.

4.2 pH-sensitive platforms for tailoring peptides and proteins release

Oscillations in the pH along the gastrointestinal tract are a big hurdle to overcome when administration of peptides and proteins is made by the oral route. Taking advantage of these variations, however, is becoming a popular approach towards the enhanced delivery of biopharmaceuticals. For example, pH-responsive systems can be tailor-made to circumvent the release of biological molecules in the stomach, while promoting site-specific release in the small or large intestine. Most of such pH-responsive systems are typically composed by polymers of anionic nature, alone or in mixture with each other or with other biomaterials, with great attention being devoted to acrylic, methacrylic and cellulose polymers (Colombo *et al*., 2009; Karimi *et al*., 2016; Rekha and Sharma, 2013). By changing the ratio between the components of the particulate systems, one can yield different release profiles (Yoshida *et al*., 2013). Polyacidic polymer chains are reticulated and condensed at low pH because their acidic groups will be protonated, and thus, unionized (Balamuralidhara *et al*., 2011). Particles can be either prepared from or simply functionalized with these pH-sensitive polymers by surface coating. When particles are exposed to biological fluids at an appropriate pH, their porosity changes and polymeric chains swell or shrink in a controllable manner in response to proton concentration, which represents the key point for the controlled release of biopharmaceuticals (Colombo *et al*., 2009). Thus, the release from the particles is largely dependent on the properties of the peptides and proteins and the swelling pattern of the polymer(s) at a specific pH (Lowman *et al*., 1999; Peppas, 2004).

Most of the pH-sensitive carriers used for oral administration of biopharmaceuticals have been used as enteric coating materials of classical pharmaceutical dosage forms and several of them have been approved by the FDA (Renukuntla *et al*., 2013; Yoshida *et al*., 2013). The most commonly used for oral delivery purposes are poly(methacrylic acid-co-methyl methacrylate) (Eudragit® L, S and F), (hydroxypropyl)methyl cellulose phthalate (HPMCP) and (hydroxypropyl)methyl cellulose acetate succinate (HPMC-AS). These polymers have different grades, corresponding to the different pH values at which the polymers are soluble (Yoshida *et al*., 2013).

Different works have already proven the efficacy of these systems in controlling the release of biopharmaceuticals in a pH-dependent manner, *i.e.*, at specific site, which resulted in their higher absorption, and hence in ameliorated biological response (Jelvehgari *et al*., 2010; Sharma *et al*., 2011). Sun *et al*. encapsulated a complex of insulin-sodium oleate into PLGA nanoparticles (213 nm), which were further encapsulated into Eudragit® FS 30D by organic spray-drying method, originating microcapsule composites of 1–5 μm (Sun *et al*., 2015). *In vitro* results revealed that drug release was pH-dependent, and *in vivo* results using a Streptozotocin (STZ) induced T1DM rat model demonstrated a relative bioavailability of

about 16%, while for free insulin it was less than 0.5%. Thus, the microcapsule composites were demonstrated to be an effective candidate for oral insulin delivery (Sun *et al*., 2015).

Shrestha *et al.* proposed an alternative system for the GLP-1 delivery, in which CS-coated PSi nanoparticles (208 nm) were incorporated into HPMC-AS nanoparticles (≈ 830 nm) by using an aerosol flow reactor (AFR) (**Figure 6**) (Shrestha *et al*., 2015). *In vitro* results indicated that there was no release of the peptide at pH 1.2, but at pH 6.8 the release was 40% after 6 h (Shrestha *et al*., 2015). When tested *in vivo* using a T2DM rat model, blood glucose levels were reduced by 32% at 8h after particles administration (Shrestha *et al*., 2016).

Other systems have also been tested. In those studies, particulate systems (either nanoparticles or microparticles) were mainly composed of positively-charged CS and negatively-charged polymer, such as Eudragit® (Jarvinen *et al*., 1998; Li *et al*., 2006), poly(γ-glutamic acid) (Sonaje *et al*., 2010a; Sonaje *et al*., 2010b; Sonaje *et al*., 2010c), alginate (Chen *et al*., 2004; Li *et al*., 2007; Mukhopadhyay *et al*., 2015), or methacrylic acid (de Moura *et al*., 2008). Sonaje *et al.*, for example, used insulin-loaded particulate systems (≈ 200 nm) made of CS and poly(γ-glutamic acid) (Sonaje *et al*., 2010b). After oral administration using a T1DM STZ-induced rat model, significant hyperglycemia occurred, and the corresponding relative bioavailability of insulin was ≈ 15% (Sonaje *et al*., 2010b). Mukhopadhyay *et al*. attempted to deliver insulin in a pH-responsive manner by using CS/alginate core-shell nanoparticles (100-200 nm) (Mukhopadhyay *et al*., 2015). *In vitro* release studies showed that almost all the encapsulated insulin was retained inside the system in simulated gastric buffer. However, sequential sustained insulin release in simulated intestinal conditions was demonstrated. When tested *in vivo,* in a diabetic mice model, the insulin-relative bioavailability was 8 % with significant hypoglycemic effects observed (Mukhopadhyay *et al*., 2015).

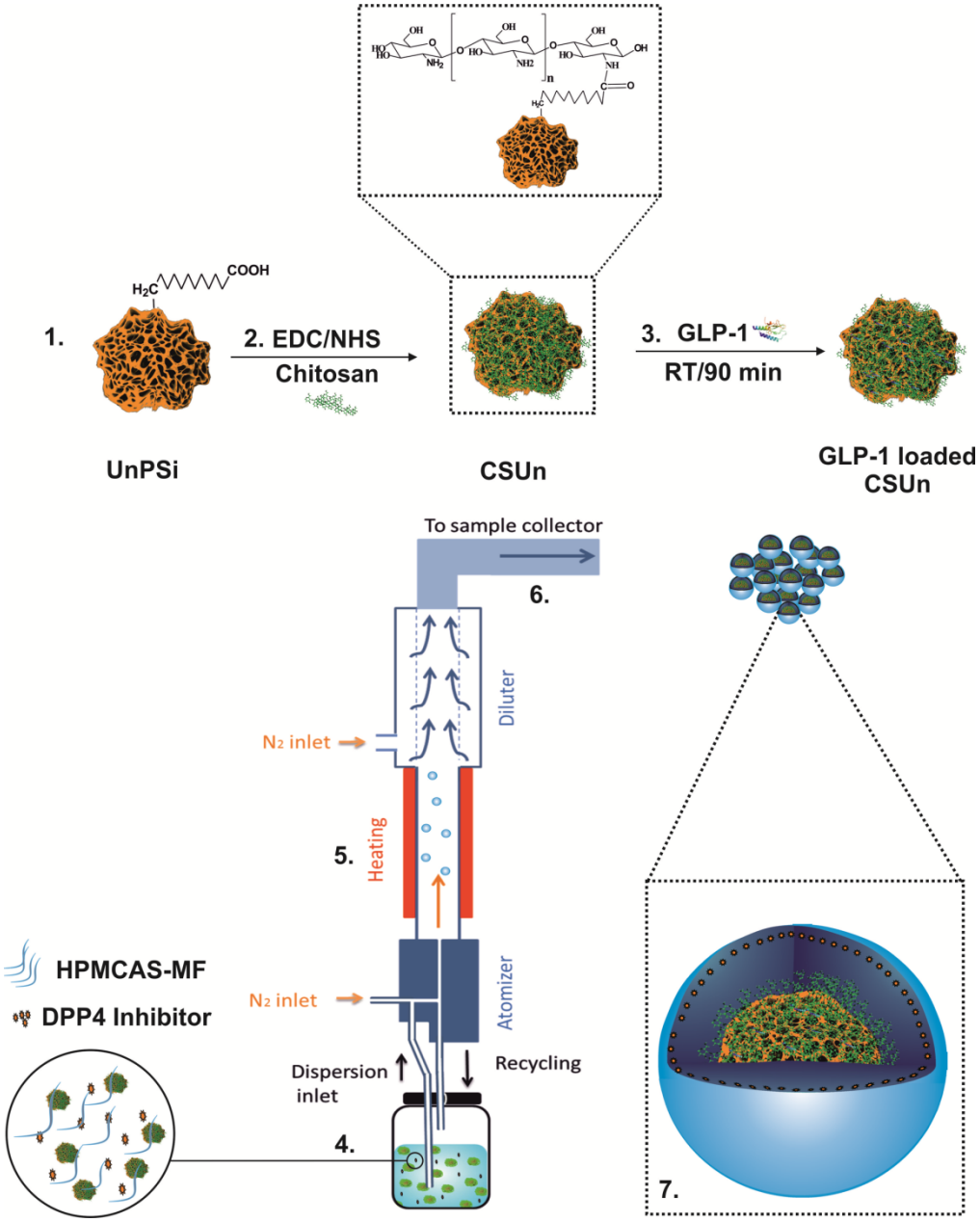

Figure 6. Schematic representation of the different steps involved in the preparation of pH-sensitive polymer coated CS-modified PSi nanoparticles (H-CSUn). The PSi nanoparticles (UnPSi) (1) are modified with CS using carbodiimide crosslinker chemistry to form CSUn nanoparticles (2). (3) Glucagon-like peptide-1 (GLP-1) is loaded into the CSUn nanoparticles by immersion technique at room temperature (RT) for 90 min. (4) The CSUn nanoparticles are dispersed in the aqueous pH-responsive polymeric solution of HPMCSAS-MF containing an inhibitor of the dipeptidyl peptidase-1 enzyme (iDPP4) to prepare the feed dispersion. The dispersion is fed into the AFR via atomizer, where is first broken down into aerosol, which is then carried out to the heating chamber by nitrogen (N_2) gas. The aerosols are dried in the

heating chamber (5), and then collected in the collection chamber (6). For synergistic therapy, along with an antidiabetic peptide (GLP-1) which was loaded into the pores of CSUn nanoparticles and iDPP4 was loaded in the matrix of the polymeric layer. (7) Schematic representation of the final H-CSUn particles (Shrestha *et al*., 2015).

4.3 Redox potential-responsive drug carriers for controlled release

The course of various cellular signaling pathways depends on the redox state of the cells and their surrounding environment (González-Flecha and Demple, 2002; Sun and Oberley, 1996). The redox state consists in the ratio of the interconvertible oxidized and reduced form of a specific redox pair (Schafer and Buettner, 2001). Several studies have, in fact, reported that the difference in potential redox level observed between intra and extracellular environments varies in several orders of magnitude, specifically <0.01 nM glutathione (GSH) for the extracellular fluid, while for the cytoplasm ranges from 1 to 11 mM of GSH (Remant *et al*., 2014; Schafer and Buettner, 2001).

Beyond the abovementioned pH-responsiveness, a new generation of drug carriers has been also endowed with stimuli-responsive features in terms of redox potential, contributing to satisfy the trend of non-invasive therapy. In this context, and taking advantage of physiological signals, redox-responsive drug carriers hold enormous potential for varying biomedical applications. The basic principle of such systems consists on the proper molecular design and related synthetic methodology to enable the utilization of distinct differences in redox potentials.

In fact, polymeric nanomaterials incorporating different redox-responsive functional groups acquire “intelligence” that allows for drug transport to the required region (Huo *et al*., 2014). Zhao *el al*., for example, engineered single-protein polymeric nanocapsules with a redox-responsible, disulfide-containing crosslinker for escorting proteins to the cytosol in a controlled release fashion (Zhao *et al*., 2011). The authors describe the non-covalent encapsulation of the target protein through *in situ* interfacial polymerization into a positively-charged polymeric shell interconnected by disulfide-containing crosslinkers (**Figure 7**). The nanocapsules were shown to be rapidly degraded when treated with physiologically relevant concentrations of GSH, being then internalized into cells and efficiently delivered functional proteins in a variety of cell lines (Zhao *et al*., 2011).

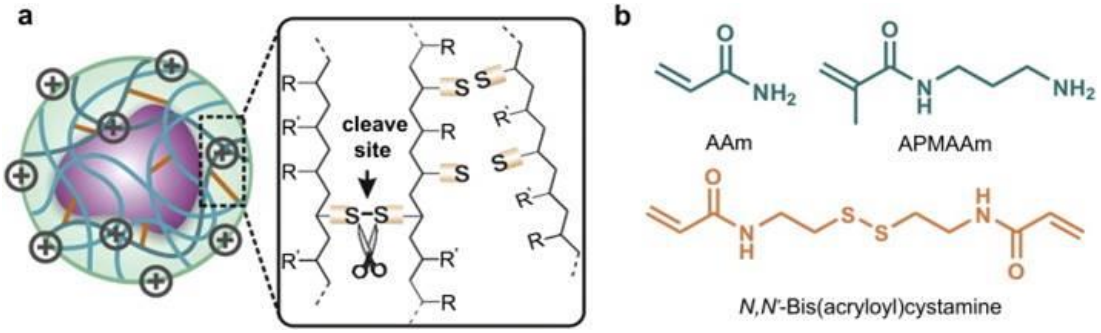

Figure 7. Formation of redox-responsive protein nanocapsules. (a) Schematic of protein nanocapsules with redox-responsive polymeric matrix (R and R' represent different monomers' moieties); and (b) Chemical structures of monomers and crosslinker for disulfide nanocapsules (Zhao *et al*., 2011).

4.4 Targeting at the absorption site: membrane transporters and receptors on the gastrointestinal tract

One of the greatest advantages in functionalizing the surface of multistage delivery systems is the possibility to tune them for selective or specific recognition of molecules or sites. In the particular case of oral delivery of peptides and proteins, this strategy allows for the direct targeting of molecules present at the small or large intestine cells, increasing the local concentration of the administered biopharmaceuticals by releasing them when the particle is bound to the target, and potentially increasing the uptake and endocytosis of orally delivered molecules (Subbiah *et al*., 2010).

Even constituting solely 1% of the total cell population, M cells are the most studied among the different cell types present at the intestinal epithelium when considering the oral delivery of particulate systems (des Rieux *et al*., 2006). Due to their physiological functions, these cells seem to be the perfect target for the uptake of carrier systems. Many potential target ligands, either specific or non-specific, have been described as expressed by M cells (des Rieux *et al*., 2006; des Rieux *et al*., 2013; Wong *et al*., 2003). Lectins, for example, are commonly used to target M cells (**Figure 8**). Lectins are a structurally diverse group of proteins and glycoproteins that reversibly and with relatively high affinity bind to carbohydrate moieties and tether mucins at cell membranes, (des Rieux *et al*., 2006; des Rieux *et al*., 2013). Due to the variability of carbohydrate expression from cell-to-cell type, the use of lectins allows targeting for specific sites within the intestinal tract. Binding of particles functionalized with lectins to M cells can occur by either adsorption or covalent coupling (Clark *et al*., 2001; des Rieux *et al*., 2006; des Rieux *et al*., 2013; Plapied *et al*., 2011). The

most used lectin is wheat germ agglutinin (WGA), known to bind to N-acetyl-D-glucosamine and sialic acid residues. Wang *et al.* conjugated WGA to the surface of PLGA nanoparticles and observed increased cytoadhesive and cytoinvasive properties when comparing to non-functionalized nanoparticles, attributed to the over expression of N-acetyl-D-glucosamine-containing glycoproteins in the colon intestinal cells (Wang *et al*., 2010). Other lectins, such as galectin 9 and sialyl Lewis A antigen, have also been described as ligands for M-cells (Pielage *et al*., 2007). Noticeably, *Ulex europaeus* agglutinin-1 (UEA-1) and *Aleuria aurantia* lectin (AAL), which are able to bind to α-L-fucose and are often considered as M cell ligands, can target some animal species, but not humans (Clark *et al*., 2001; D'Souza *et al*., 2012; Gupta *et al*., 2013). These are non-conserved inter-species epitopes, losing thus their appeal for clinic use.

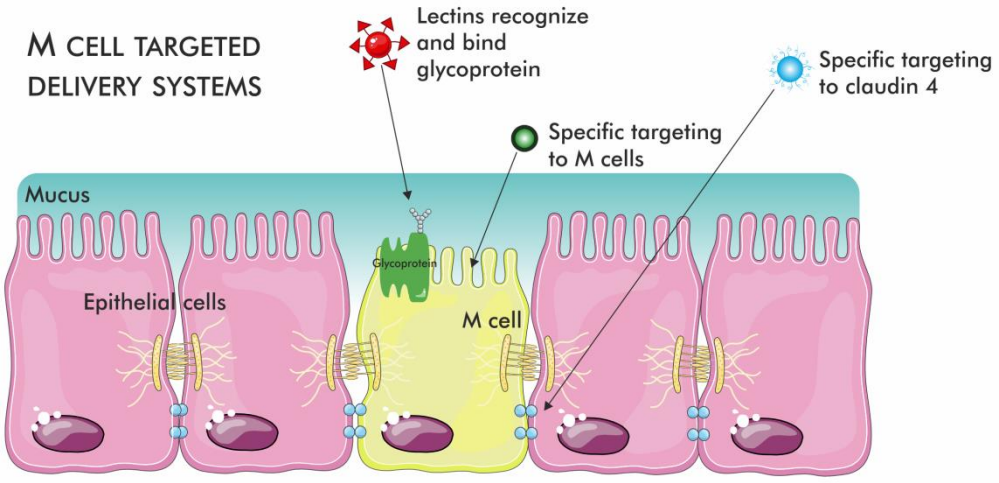

Figure 8. Strategies for surface modification of carrier systems targeting M cells, including lectin-anchored systems that specifically recognize glycoproteins expressed in the surface of M cells, specific targeting though carrier coating and specific targeting to the transmembrane receptor claudin 4. Figure produced using Servier Medical Art.

Another strategy for targeting M cells is related to their own function. M cells are responsible for presenting pathogens to neighboring immune cells through endocytic pathways (des Rieux *et al*., 2013). Therefore, it has been hypothesized that functionalization of carrier systems with microbial molecules might also lead to increased cell uptake (Clark *et al*., 2001). In this context, coupling microbial adhesins from bacterial species, such as protein invasion *(Yersinia)* and long polar fimbria (LPF) (*Salmonella),* to the surface of particulate systems would favor their internalization in the intestinal mucosa (Clark *et al*., 2001; des Rieux *et al*., 2006; Pielage *et al*., 2007). Moreover, specific viral proteins or peptides responsible for M cell adhesion, such as those expressed in the reovirus (8 haemagglutinin

σ1 protein) can be attached to particles in order to target M cells (Clark *et al*., 2001). In a similar approach, Rajapaksa *et al.* produced sub-micron sized PLGA particles incorporating influenza hemagglutinin with or without the c-terminal targeting peptide *clostridium perfringens enterotoxin* (CPE)30, which is known for its binding ability to claudin 4, another transmembrane protein highly expressed in M cells (Rajapaksa *et al*., 2010). The authors observed increased uptake of targeted nanoparticles in both *in vitro* and *in vivo* uptake studies. Thus, targeting particles to claudin 4 can also be considered as a potential strategy to ultimately increase biopharmaceuticals uptake (**Figure 8**) (Lo *et al*., 2012; Rajapaksa *et al*., 2010).

M cells apical membrane receptors have also been suggested as other promising targeting molecules for oral delivery of biopharmaceuticals. Cholera toxin (Ctx) and its derivatives, for example, are frequently co-administered with mucosal antigens in order to enhance immunogenicity (Mestecky *et al*., 1997; Williams *et al*., 1999). In fact, these mucosal adjuvants display high affinity to its non-toxic B subunit (CtxB) present in Ganglioside GM1 receptor of the apical membranes of M cells (Clark *et al*., 2001; des Rieux *et al*., 2006; Fievez *et al*., 2009). Consistently, Haropakis *et al.* demonstrated amplified effectiveness of liposomes as antigen delivery systems after conjugation with recombinant CtxB following oral administration in mice (Harokopakis *et al*., 1998). Several specific pathogen recognition receptors (PRR), also present and overexpressed at the apical membrane of M cells, such as platelet-activating factor receptor, Toll-like receptor-4 (TLR-4) (Kyd and Cripps, 2008), glycoprotein 2 (GP2) (Kim *et al*., 2012) and α5β1 integrin (Kyd and Cripps, 2008) could serve the same purpose (des Rieux *et al*., 2006; Plapied *et al*., 2011). Thus, the use of peptides, such as Arg-Gly-Asp (RGD) and Leu-Asp-Val (LDV) or peptidomimetics (RGDp and LDVp), can be useful ligands for targeting these receptors aiming for ameliorated strategies for increased uptake of orally delivered biopharmaceuticals (Fievez *et al*., 2009; Mundargi *et al*., 2008). Interestingly, previous studies have reported the use of such ligands to functionalize the surface of PLGA and PLGA-based nano- and micro-particles with encouraging results (Fievez *et al*., 2009; Mundargi *et al*., 2008).

Similarly to M cells, enterocytes have also been investigated for targeting at the absorption site. Enterocytes are the main responsible for nutrient absorption and are the most abundant cells in the small intestine, being therefore an immediate target for particulate systems. As in the case of M cells, lectins are also moieties that can be used for targeting enterocytes. WGA, tomato lectin (TL) and concanavalin A (ConA) are examples of such lectins; WGA and TL bind specifically to N-acetyl-D-glucosamine, whereas the ConA binds to α-D-mannose residues (des Rieux *et al*., 2013; Zhang *et al*., 2005).

Molecules that bind specifically to receptors of the apical membrane of enterocytes, such as biotin, vitamin B12, vitamin B1 (thiamide) and folic acid, reveal to have promising

features for developing strategies to achieve absorption and/or particle uptake by enterocytes (Roger *et al*., 2012; Simons and Fuller, 1985; Youn *et al*., 2008). Roger *et al.* evaluated the effect of folic acid functionalization on transcellular transport of PLGA nanoparticles (213 nm), and observed an 8-fold higher transport compared to the free therapeutic agent tested (Roger *et al*., 2012). Interestingly, confocal microscopy studies revealed that the functionalized particles internalized by the cells did not compromise the integrity of the tight junctions, reinforcing the potential of this system to enhance the absorption of drugs with poor oral bioavailability (Roger *et al*., 2012).

Other receptors, such as TLR-4 or the neonatal Fc receptor (FcRn), can be a targ*et al*so for enterocytes (Neal *et al*., 2006). FcRn is receiving particular attention over the last years for therapeutic applications, since it naturally mediates the transport of immunoglobulin G (IgG) antibodies across epithelial barriers, by interacting with the Fc portion of IgG (Martins *et al*., 2016). Recently, Pridgen *et. al.* described a PLA-PEG block copolymer-based particulate system (55 nm), loaded with insulin and functionalized with an Fc-thiol surface ligand groups to target the FcRn (**Figure 9**) (Pridgen *et al*., 2013). After oral administration of Fc-decorated nanoparticles to mice, a prolonged hypoglycemic response was elicited in wild-type animals, superior to that observed in animals treated with untargeted systems. Also interestingly, hypoglycemia was absent in FcRn knock-out mice (Pridgen *et al*., 2013).

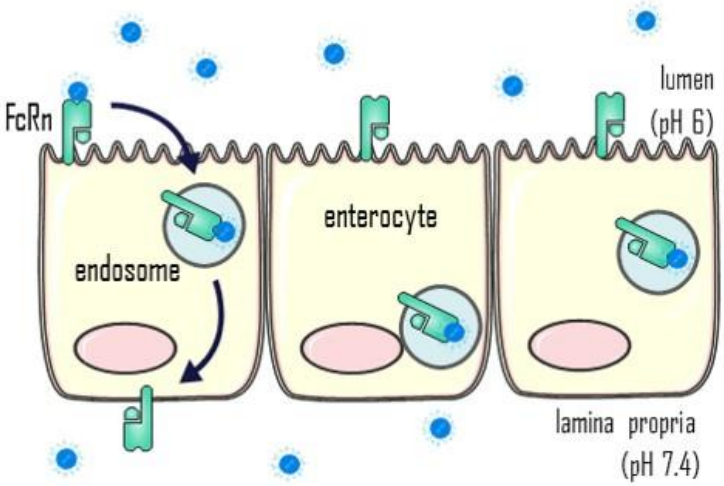

Figure 9. Schematic of Fc-targeted nanoparticle transport across the intestinal epithelium by the FcRn through a transcytotic pathway. IgG Fc on the nanoparticles surface binds to the FcRn on the apical side of absorptive epithelial cells under acidic conditions in the intestine, nanoparticles-Fc are then trafficked across the epithelial cell through the FcRn transcytosis pathway in acidic endosomes. Upon exocytosis on the basolateral side of the cell, the physiological pH causes IgG Fc to dissociate from the FcRn, and nanoparticles-Fc are free to

diffuse through the intestinal lamina propria to the capillaries or lacteal and enter systemic circulation. Figure produced using Servier Medical Art.

Another approach to consider for achieving specific target of particulate systems could be the use of receptors of bacteria present within the intestinal flora. For example, enterocytes can be targeted by the conjugation of Ctx or F4 fimbriae to particulate systems. Ctx is a protein complex which binds to GM1 gangliosides, being also present on the surface of enterocytes, and F4 fimbriae allow the microorganisms to adhere to F4-specific receptors present on the brush borders of villi (Melkebeek *et al*., 2012; Van den Broeck *et al*., 2000).

Among enteroendocrine cells, L cells are the most promising targets for functionalized carrier systems. Plenty of lipidic ligands can bind to their various surface-expressed, G-protein-coupled receptors (GPCRs), namely GPR109a, GPR119, GPR120 and GPR40, and could serve as targets for coated particles (des Rieux *et al*., 2013). TGR5 receptor (also known as M-BAR, GPBAR-1 or GPR131), also belonging to the GPCRs superfamily, could also be targeted by bile acids, and therefore, promote increased cellular uptake of functionalized particles (des Rieux *et al*., 2006).

Even representing the second most abundant cell type in the intestine and the responsible for mucus production, goblet cells are not a common choice for targeting. In fact, most strategies involving goblet cells are usually based on establishing interactions with mucins present at mucus. For instance, Jin *et al.* used the peptide CSKSSDYQC (CSK), which shows affinity to the mucus released by these cells, to modify particulate systems (318 – 342 nm) and allow them to use goblet cells as their specific gateway (Kang *et al*., 2008). Nevertheless, Jin *et al.* extensively investigated the effects of targeting nanoparticles to goblet cells and the influence of the functionalization if oral absorption of insulin (Jin *et al*., 2012). For this purpose, the authors developed insulin loaded nanoparticles using trimethyl CS chloride (TMC) modified with CSK. In summary, *in vitro*, *ex vivo* and *in vivo* studies revealed that CSK functionalized particles enhanced the uptake of the TMC nanoparticles, even when the targeting recognition was partially affected by mucus, and increased the insulin permeation across the epithelium. Additionally, the modified particles induced a significant increase in insulin internalization via clathrin and caveolae mediated endocytosis on the goblet cells (Jin *et al*., 2012). Consistently, better hypoglycemic effects were observed in diabetic rats after oral administration of the CSK-TMC nanoparticles when compared to the results obtained from the administration of unmodified particles.

The examples described within this section are still at early preclinical stages and have been developed mostly by academia. However, several technologies are also being developed by the pharmaceutical industry towards the oral delivery of biopharmaceuticals

using intestinal targeted systems. TrabiOral™ (Transgene Biotek Ltd, India), for example, is a platform using SLN functionalized with an active ligand claimed to be specific for a previously undescribed intestinal transporter. It has been suggested that targeting this receptor may be advantageous when compared to vitamin B12 and transferrin receptor targeting, namely due to its ability to provide relatively higher uptake, as well as the low cost and versatility of ligand conjugation (Aguirre *et al*., 2016). Similarly, the same company has another patent that claims a surface modification of lipid nano/micro particles containing WGA as a ligand, which targets the particulate systems to the intestine (Rao, 2008).

4.5 Co-delivery of biopharmaceuticals with other modulators

A final alternative strategy to maximize drug systemic bioavailability after oral administration consists in the concomitant delivery of peptides and proteins with other modulators, such as absorption enhancers or enzymatic inhibitors. In spite of not being considered as a direct functionalization of drug delivery systems, the inclusion of modulators in the formulation of nanoparticulate systems holds potential as a parallel strategy to enhance oral absorption of drugs with non-optimal delivery properties. Several modulators have already been tested and are therefore reviewed in this section.

4.5.1 Absorption enhancers

Absorption enhancers may be considered functional excipients, which can be included in the formulation of particulate systems to improve the absorption of orally delivered peptides and proteins (Aungst, 2012). These compounds have been investigated as such since the 1960s and, as suggested by their name, are recognized for increasing the transport of the molecules through the epithelial barriers. Enhancement can be reached by either of two mechanisms: (i) increasing paracellular permeability, due to a slight and reversible modification of tight junctions conformation, which leads to larger paracellular spaces, and thus, less steric hindrance, or (ii) increasing the transcellular permeation, by which disruption of the packing of membrane lipids and changes in membrane fluidity lead to the modification of the cell membrane structural integrity (**Figure 10**) (Aungst, 2012; Kondoh and Yagi, 2007; Maher *et al*., 2016). In all cases, these approaches can compromise mucosal membrane barriers and thus can be considered potentially toxic (Maher *et al*., 2009), especially in the case of long-term usage. Chronic administration of absorption enhancers may cause serious side effects such as permanent damage to the intestinal

epithelium and undesirable passage of certain toxins, viruses and pathogens across the intestine (McCartney *et al*., 2016; Scott Swenson and Curatolo, 1992).

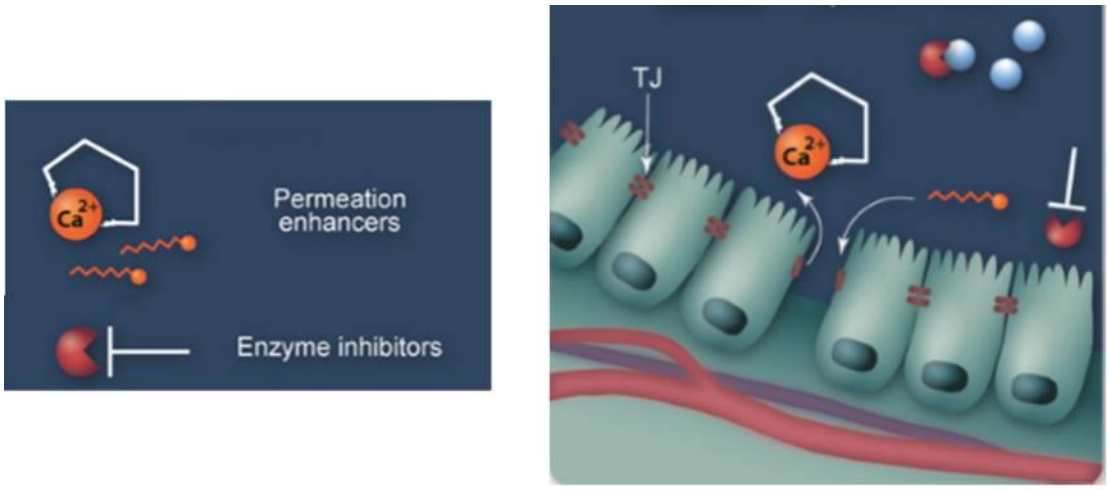

Figure 10. Strategies for oral delivery of peptides and proteins that act on local or systemic targets. Absorption enhancers and enzymatic inhibitors can also be included in the formulation to further improve gastrointestinal resistance. To achieve meaningful systemic exposure, absorption enhancement by tight junction-disrupting excipients is often needed. Adapted from (Moroz *et al*., 2016).

Some of the most traditional absorption enhancers with prominent effects are bile salts, fatty acids, chelating agents (form complexes with calcium ions, leading to tight junction rupture (Aungst, 2000; Park *et al*., 2011)), zonula occludins toxin (ZOT) peptide (Lee *et al*., 2016), and polymers like CS (Lee *et al*., 2016) or thiolated polymers (Kondoh and Yagi, 2007). Particulate systems themselves can also act as absorption enhancers if surfactants are used during their production (Aungst, 2000), or due to their possible lipophilic nature, which is able to promote pore formation and damage the integrity of cell membranes (Lee *et al*., 2005; Leone-Bay *et al*., 2001).

Short peptide sequences, known as cell-penetrating peptides (CPP), have also been described for acting as absorption enhancers when conjugated with particulate systems (Wang *et al*., 2014). Originally considered as a “Trojan horses”, CPPs have the ability to enter cells and increase transcellular transport without causing damage or eliciting a cellular response (Palm-Apergi *et al*., 2009; Shi *et al*., 2014; Wang *et al*., 2014). CPPs can be divided according to their nature into cationic peptides, such as HIV-1 Tat and oligoariginine, or amphiphilic peptides, such as penetratin. Different from ligands described in the previous

section, CPPs have the ability to increase the permeability of various types of cells with no specificity for one particular type over the others (Amin *et al*., 2015). A study made by Guo *et al*. (Guo *et al*., 2016) showed that the cellular uptake amount and transcellular transportation performance of PLGA nanoparticles modified with CS and a Tat CPP were enhanced compared with those of without CS and plain particles. The efficacy evaluation, using a diabetic rat model, demonstrated that the hypoglycemic effect of Tat-CS functionalized nanoparticles, loaded with insulin, was 6.89 times higher than that of plain particles and 1.79 times higher than the CS modified particles (Guo *et al*., 2016).

Due to the positive charge of some CPPs, zeta-potential is thought to be one of the possibilities for their role in cellular uptake. However, even particulate systems which are conjugated with CPP and have negative zeta-potential still present effective cellular uptake. The mechanism through which CPP enter the cells is still unclear, but there is a consensus in two major mechanisms: direct penetration and endocytosis (Barany-Wallje *et al*., 2005; Christiaens *et al*., 2004). Nevertheless, CPPs like any other peptide in general, suffer from shortcomings, such as typical short duration of action.

Considering all of the above discussed features of absorption enhancers, only few of them are presently in use in clinical practice due to the difficulty of developing enhancers with high specificity and low toxicity. Though not based in the use of nano or micro particulate systems, there are already products in clinical trials or even in the market using absorption enhancers, showing their positive role in the absorption of biopharmaceuticals that may be promising candidates to use in further developments of particulate systems for oral delivery of biopharmaceuticals (Aungst, 2012; Choonara *et al*., 2014; Ding *et al*., 2004; Kidron, 2007; Luzio *et al*., 2010; Maher *et al*., 2009; Maher *et al*., 2016; Tillman *et al*., 2008).

4.5.2 Enzymatic inhibitors

Proteins and peptides are highly susceptible to degradation by several enzymes present in the gastrointestinal tract, as previously discussed. Therefore, inhibiting these degrading enzymes can be an interesting approach to increase the amount of biologically active molecules that can reach the intestinal cell layer and may undergo absorption (**Figure 10**). Selection of the appropriate enzymatic inhibitor is entirely dependent upon the structure and nature of the peptide or protein to be delivered and its overall metabolism (Pawar *et al*., 2014). Common examples of intestinal protease inhibitors include: aprotinin (inhibitor of trypsin and chymotrypsin), soybean trypsin inhibitor (inhibitor of pancreatic endopeptidases), FK448 (chymotrypsin inhibitor), and chicken ovomucoid (trypsin inhibitor) (Choonara *et al*., 2014). The inhibitors may be further divided regarding their nature in either aminoacid-based

or non-aminoacid-based inhibitors. Those based on aminoacids bind in a competitive way to the active site of the enzyme and may not be completely effective due to their rapid dilution, digestion and/or absorption along the gastrointestinal tract (Bernkop-Schnurch, 1998; Moroz *et al*., 2016). One example of the use of aminoacid-based enzymatic inhibitors is the study performed by Marschutz *et al*. (Marschutz and Bernkop-Schnurch, 2000). This group developed a carrier system for the oral delivery of insulin using elastatinal and Bowman–Birk inhibitors covalently bound to sodium carboxymethylcellulose (Na-CMC), a mucoadhesive polymer. They showed that the activity of trypsin and chymotrypsin was inhibited to a great extent with only 22.3 ± 2.5% (mean ± SD, n=3) of peptide degradation in comparison with the system without inhibitor, in which all of the drug was metabolized after 1 h (98.7 ± 0.4%) (mean ± SD, *n*=3) (Marschutz and Bernkop-Schnurch, 2000). In another work, Werle *et al*. (Werle *et al*., 2007) produced a carrier system by covalently attaching a trypsin/chymotrypsin inhibitor (aprotinin) to CS. These conjugates were proven to protect proteins against proteolytic enzymes *in vitro* through enzyme assays and *in vivo* using a rat model. Here, the mean blood glucose level was decreased to 84 ± 6% 8 h after administration, in contrast to the control where the mean blood glucose level increased to 121 ± 8% of the initial measured blood glucose level (Werle *et al*., 2007).

Besides the use of inhibitors that directly bind to enzymes and reduce or hinder their activity in a reversible or irreversible way, metabolism of active peptides and proteins may also be preventable, in an indirect fashion, by using modulators that change pH. Modifying proton concentration to values that inhibit specific enzymes may be considered for protecting active biopharmaceuticals, such as in the case of using citric acid, which changes the pH from 6 to 3 (Aungst, 2012; Friedman and Amidon, 1991; Moroz *et al*., 2016; Welling *et al*., 2014). As in the case of absorption enhancers, the long term use of enzyme inhibitors may also cause some toxic events at the gastrointestinal tract and induce non-specificity in action via altering the normal metabolic pathways, which may lead to delayed digestion of proteins from the normal diet, intestinal mucosal damage and feedback-regulated protease secretion (Choonara *et al*., 2014; Moroz *et al*., 2016; Pawar *et al*., 2014).

Considering the pros and cons of the co-delivery of peptides and proteins with a single modulator, either an absorption enhancer or an enzymatic inhibitor, it seems that a combination of both may prove to be more feasible in improving oral bioavailability (Araújo *et al*., 2012). Enteris BioPharma Inc. (Boonton, New Jersey, United States) has developed the clinically tested Peptelligence™ technology, which comprises an enteric-coated tablet with two key components: a permeation enhancer and an enzyme inhibitor. These excipients are released in the small intestine, where the permeation enhancer loosens tight junctions between the intestinal epithelial cells and the enzyme inhibitor transiently decreases the intestinal pH in order to inactivate the enzymatic activity. As an example of the application of

Peptelligence™ technology for the oral delivery of biopharmaceuticals, Unigene Laboratories Inc. (New Jersey, USA) scientists formulated salmon calcitonin as an enteric-coated tablet, choosing lauroyl L-carnitine as preferred permeation enhancer. The company licensed the developed formulation of oral calcitonin to Tarsa Therapeutics Inc. (Philadelphia, USA), and the product TBRIA™ is currently in late-stage clinical trials for the treatment and prevention of postmenopausal osteoporosis in collaboration with Novartis (Crotts *et al*., 2002).

5. Microfluidics for multisystems platforms production

Traditional methos are still extensively employed in the preparation of multistage platforms based on particulate carrier systems. However, due to the lack of precision in the control of the production processes, these methods have faced critical challenges, such as the polydisperse size distribution and batch-to-batch variability, which hinder their scale-up. Moreover, frequently these methods require personal staff while doing the production of the systems, with a high amount of lab glassware and other equipment needed and with the produced batches having low yields (Kim *et al*., 2012; Liu *et al*., 2013b).

Hence, biomaterials science together with physics, chemistry and nanotechnology sciences, among others, took a step forward in the the development of miniaturized systems for drug delivery applications (Craighead, 2006; DeMello, 2006; Valencia *et al*., 2012; Yager *et al*., 2006). These miniaturized systems, with few square centimeters, offer several advantages over the traditional macroscale systems for the production of particulate carriers, shrinking thus traditional bench systems (Mazzitelli *et al*., 2013; Janasek *et al*., 2006).

Microfluidics is one of these miniaturized systems and gained a lot of popularity due to the possibility of manipulating nanoliter and sub-nanoliter volumes in microscale fluidic channels, with dimensions of tens to hundred micrometers (Valencia *et al*., 2012; Whitesides, 2006). Using the microfluidic technique it is possible to assemble different biomaterials towards the production of multistage platforms. Microfluidics offers an effective control over the characteristics of produced systems, leading to a narrow size distribution and high batch-to-batch reproducibility. Moreover, it has the advantage of encapsulate different cargos with a theoretical efficiency of 100% (Valencia *et al*., 2012; Yang *et al*., 2012).

Very briefly, the preparation of emulsions through microfluidics involves the injection of the dispersed phase into another immiscible or partially immiscible continuous phase, through a specially design microfluidics device. The most commonly used geometries are the T-junction (Garstecki *et al*., 2006; Murshed *et al*., 2009), co-flow (Cramer *et al*., 2004; Xiong *et al*., 2007), cross-flow (Xu *et al*., 2005; Wagdarea *et al*.) and flow focusing (Schmid and

Franke, 2013; Yobas *et al*., 2006). These devices can be also fabricated using four different materials as glass capillaries (Duncanson *et al*., 2012), polydimethylsiloxane (PDMS) (Whitesides, 2006), metal (Görke *et al*., 2005) or poly(methyl) methacrylate (Ogilvie *et al*., 2010).

Since in the work described below, only microchips made with glass capillaries with a flow-focusing geometry were used, a focus on these systems seems relevant in relation to the others. These chips have a real three-dimensional geometry and consist of the coaxial assembly of a series of glass capillaries on glass slides. Glass capillaries present the advantages of having chemical resistance, excellent optical properties, low electric conductivity, smooth surface and easily and precisely controlled surface wettability (Duncanson *et al*., 2012; Shah *et al*., 2008; Vladisavljević *et al*., 2013). For the production of single emulsion particles in the flow-focusing geometry devices, the dispersed phase flows inside the square capillary and the continuous phase flows between the square and round capillary in the opposite direction (**Figure 11**). The dispersed phase is focused by the continuous phase through the narrow orifice of the tapered round capillary, which is known as hydrodynamic flow-focusing. The continuous phase exerts pressure and shear stress that force the dispersed phase into a narrow thread, which breaks inside and downstream of the orifice and generates droplets in the collection capillary. A major advantage of these geometry devices is that smaller particles can be produced comparing with the other geometries (Schmid and Franke, 2013; Yobas *et al*., 2006).

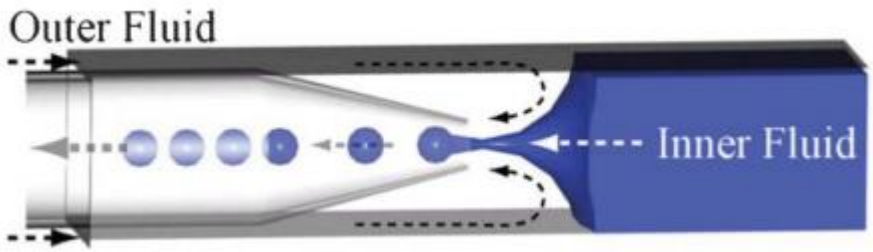

Figure 11. Microfluidic strategy of a flow-focusing capillary device for making single emulsion droplets (Duncanson *et al*., 2012).

6. Safety, toxicity and regulatory concerns of multisystems platforms based in particulate carrier systems

Materials and thereof products require to be proven safe and compliant with the requirements of healthcare regulatory bodies in order to be considered for medical use. Nevertheless, issues regarding the safety and toxicity of materials and how these may affect human health and the environment have been raised over the years (Austin and Lim, 2008; Bimbo *et al*., 2012; Klaine *et al*., 2012; Nel *et al*., 2006; Sharifi *et al*., 2012). It seems clear, however, that adverse effects are dependent on the nature and type of usage of materials, as well as on the route, dose and duration of exposure (Bimbo *et al*., 2012; Oberdörster *et al*., 2005). Various tools and techniques for assessing the toxicity of materials were developed over the last decades in order to address specific necessities in the medical field. Still, standards for the assessment of the toxicity of materials and compendia methods are eagerly needed (Alkilany *et al*., 2016; Arora *et al*., 2012), which, in turn, may be partially responsible for the slow pace towards the approval of new products, as discussed below.

Carrier systems are typically produced with biodegradable, biocompatible and low toxicity materials allowing for overall safety (Elsabahy and Wooley, 2012). Nevertheless, using materials previously demonstrated as safe does not necessarily translate into the same outcome when used at different scales, such as the nanoscale (Bartlett *et al*., 2015). The specific engineered surface of the carrier systems and its functionalization may help modifying the original properties of these materials and improve biodegradability, biocompatibility and toxicity profiles (Bianco *et al*., 2011; Bimbo *et al*., 2012; Liu *et al*., 2011; Nurunnabi *et al*., 2013; Shahbazi *et al*., 2013). However, the functional moieties incorporated into the systems may present intrinsic bioactivity and, possibly, relevant toxicity. Their evaluation must, therefore, consider possible biological effects while non-associated to systems, as well as after being released upon disassembly, cleavage or degradation/biotransformation of the systems.

The dimensions and surface properties of carrier systems seem to particularly affect their ability to distribute and accumulate in the human body, but that alone may not be determinant of any deleterious effects in health (Kulkarni and Feng, 2013). Other important factors, such as solubility, biodegradability, biocompatibility and inherent toxicity of used materials, as well as processing, stability, physical state, agglomeration, geometry, porosity and surface chemistry of produced carrier systems need to be taken into account (Luyts *et al*., 2013; Podila and Brown, 2013; Yu *et al*., 2012). The routes by which particulate systems are intended to be administered will also impact their interactions with biological systems (Fu *et al*., 2013). In the particular case of oral delivery, the administration of biopharmaceuticals must be done in much higher amounts than in a subcutaneous route. Such amounts in the

intestine may raise toxicological problems that the majority of investigations usually neglect or underestimate (Fonte *et al*., 2015).

From a pharmaceutical perspective and in a broader sense, materials (apart from active pharmaceutical ingredients) used in the design and production of carrier systems may be considered as excipients (Bansal, 2014). Therefore, their evaluation should comply with general requirements for toxicity and safety evaluation of common excipients as defined by different regulatory authorities (Elder *et al*., 2016; Osterberg *et al*., 2011). Thus, general statements on specific materials and delivery routes are not straightforward and a case-by-case analysis supported with consistent experimental data is highly recommended (Hoet *et al*., 2009).

Due to the nature and particular complexity of carrier systems, regulators have been considering and discussing specific guidance for multistage platforms-based products intended to be used in human and veterinary medicine. Despite of that, there is a stringent lack of usable regulations. The debate has been intense and, in most cases, inconclusive (Satalkar *et al*., 2015). The FDA has long been engaged in promoting various endeavors in order to establish standards and rules for such products (Sadrieh and Espandiari, 2006). Despite of these and following efforts in establishing new and specific regulations, review processes of material-containing products have been essentially conducted over the years in a similar way as those for “conventional” medicines through the Center for Drug Evaluation and Research. The FDA considers that current regulatory pathways are robust but flexible enough, thus abbreviating any urgent need for additional regulation (Tyner *et al*., 2015) and chosen deliberately to work mostly on a product-orientated fashion (Hamburg, 2012). Indeed, available specific guidance on particulate-based medicinal products from the FDA is still quite ambiguous and, in many cases, without binding character or still in draft form.

Strategies for the regulatory approval of particular product by the FDA are well documented at the agency website. Still, there is some controversy concerning this issue, namely when drugs and devices are associated (Bartlett *et al*., 2015). Apart from the general guidance on whether a product involves the application of nanotechnology, the only other available (draft) guidance concerning particulate-based systems addresses liposome drug products and polymer-coated superparamagnetic iron oxide nanoparticles (SPIONs). This lack of specific regulation and active guidance has been interpreted by some experts has potentially harmful to the development and market introduction of new products (Bawa, 2011).

These systems also require complying with current European legislation on medicinal products. EMA has issued a few Reflection Papers on that over recent years but guidelines are still missing. Different products already approved by this agency have been the focus of such guidance, including those based on liposomes (*e.g.*, Caelyx® and MEPACT®), albumin

nanoparticles (Abraxane®), SPIONs (Sinerem®) and nanocrystals (Rapamune®). Still, recommendation whether or not a new product should be considered safe is based on a case-by-case analysis. The European agency also recognizes the fast trends in the field towards increasingly complex products, and is already looking at next-generation products (de la Ossa, 2015). Various information on past approvals is also available from EMA, namely in the form of European public assessment reports. It should be stressed that efforts for regulatory issues are not an exclusive of the FDA and EMA and that many other agencies are actively engaged in sorting out this enduring gap (Bartlett *et al*., 2015; Guo *et al*., 2014; Tinkle *et al*., 2014).

The road ahead seems bumpy and various challenges face the regulation of technology-based medicinal products. Despite of the novelty and complexity as compared to "conventional" medicinal products, currently approved products are still relatively simple and increasing regulatory issues are expected when it comes to systems comprising multiple active agents and functionalities. Another huge challenge for further regulation concerns the development of analytical tools that can help establishing which and how physicochemical properties of materials will impact safety and effectiveness of medicinal products (Bartlett *et al*., 2015; Jiang *et al*., 2011; Tyner *et al*., 2015). It is not hard to expect that the greater the complexity of developed systems, namely when facing surface functionalization, the most difficult it will be to define and standardize characterization techniques. Different worldwide agencies are aware of the coming hurdles regarding the approval of such products and extensive advances in regulations is urgently needed (Bowman and Gatof, 2015). Overall, analytical tools and mandatory testing should assure that materials used throughout the life cycle of a medicinal product are consistent in terms of their properties defining quality, safety and efficacy (Tyner *et al*., 2015). This should, however, allow facilitating the development process of new products and not constitute overwhelming barriers towards approval, particularly regarding the cost associated with developing technology-based products and the typical typology of applicants (small to medium sized companies). Current lack of international standardization and harmonization may stand in the way of such goal, even if small but important steps have been taken in order to abbreviate such hurdles. For example, procurement of parallel advice from EMA and FDA is currently possible. Furthermore, on-going efforts are being made in order to establish bridging between global regulators and with other scientific, economic and political organizations, moving forward through convergent pathways (Bartlett *et al*., 2015). Coordination between different agencies will be crucial in order to achieve harmonization and boost the development of new surface functionalized materials for the oral delivery of biopharmaceuticals (Sainz *et al*., 2015).

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CHAPTER II

Overview and Aims

1. Overview

Research on nanotechnology has become considerably relevant in healthcare, where the potential benefit for patients is high. A focus on diseases with the most relevant socio-economic impact, such as diabetes *mellitus*, is needed. Due to its chronic nature and high prevalence, diabetes *mellitus* has become one of the main burdens for modern society.

Peptides and proteins, due to their exquisite specificity, typically with no adverse effects and low interference with other biological processes, are important in the management of this disorder. Such activity and effectiveness cannot be, in general, mimicked by conventional small-molecule drugs. However, despite the fact that the oral route is by far the most desirable way for drug administration and classically the first goal for any new active molecule, the poor oral bioavailability (usually less than 1–2%) of peptides and proteins has been severely compromising their therapeutic outcome. When compared to simple peptide solution formulations, peptide-loaded nanoparticles are less degradable, resulting in an increased bioavailability and accumulation in the required site of action, with less harmful side-effects. These particulate-based drug delivery systems may have other benefits, such as modifiable surface properties to acquire tailored cellular interactions, dual drug delivery for synergistic therapeutic outcome, pH-responsiveness that allows protection from the harsh gastric conditions, as well as site specific controlled drug release.

In the framework of this thesis, a collaborative work between the Institute for Research and Innovation in Health (i3S) at the University of Porto (Portugal) and the Faculty of Pharmacy of University of Helsinki (Finland) was established. The i3S group has a vast experience and expertise in the formulation of several advanced delivery systems for biomolecules and drugs, including peptides and proteins, using lipid and polymer-based particulate carrier systems. The main interests of the group are to try to overcome the physiological barriers and to develop increasingly “smart” systems to efficiently and in a target way delivery drugs, decreasing the effects in the surrounding tissues. The group from the University of Helsinki is internationally reputed for their expertise in porous silicon particulate carrier systems for drug delivery applications, having consistently demonstrated their effectiveness in improving the properties of the drugs. Their research interest areas are also immunotherapy, diagnosis and bio-imaging, heart diseases and diabetes and obesity. The combined expertise and close collaboration between these groups constituted a great benefit to carry out the work reported in this thesis, dedicated to developing and exploring different biomaterials to prepare new formulations for the oral delivery of GLP-1.

2. Aims

The main aim of this thesis project was to develop safe particulate-based carrier systems and evaluate they efficiency in the encapsulation of GLP-1 and in the improvement of its oral bioavailability. The ultimate objective was to use the developed system as a therapy for T2DM.

The specific objectives of this thesis were:

i) To investigate the influence of distinct biomaterial compositions on the development of nanoparticles for the encapsulation of GLP-1, on their efficiency as the carrier systems and in interacting with intestinal cells and on enhancing GLP-1 permeability across an intestinal monolayer (Chapter III).

ii) To improve the most promising delivery system previously developed in terms of intestinal cell targeting and penetration by further modifying its surface, as well as in terms of availability of delivered GLP-1 by co-loading a DPP4 inhibitor (which prolongs GLP-1 half-life) for dual-drug release, and studying the site-specific intestinal delivery, the cellular interactions and intestinal GLP-1 permeation across an intestinal monolayer (Chapter IV).

iii) To assess *in vivo* the ability of the multistage platforms developed to reduce glycaemia in a T2DM non-obese rat model (Chapter V and Appendix C).

CHAPTER III

The impact of nanoparticles on the mucosal translocation and transport of glucagon-like peptide-1across the intestinal epithelium

This chapter was based in the following published paper:

- **Araújo, F.,** Shrestha, N., Shahbazi, M.A., Fonte, P., Mäkilä, E.M., Salonen, J.J., Hirvonen, J.T., Granja, P.L., Santos, H.A. and Sarmento, B. 2014. The impact of nanoparticles on the mucosal translocation and transport of GLP-1 across the intestinal epithelium. Biomaterials. 35(33):9199-207.

1. Abstract

GLP-1 is an incretin hormone that is in the pipeline for T2DM therapy. However, oral administration of GLP-1 is hindered by the harsh conditions of the gastrointestinal tract and poor bioavailability. In this study, three nanosystems composed by three different biomaterials (poly(lactide-co-glycolide) polymer (PLGA), Witepsol E85 lipid (solid lipid nanoparticles, SLN) and porous silicon (PSi) were developed and loaded with GLP-1 to study their pemeability *in vitro*. All the nanoparticles presented a size of approximately 200 nm. The nanoparticles' interaction with the mucus and the intestinal cells were enhanced after coating with CS. PSi nanosystems presented the best association efficiency (AE) and loading degree (LD), even though a high AE was also observed for PLGA nanoparticles and SLN. Among all the nanosystems, PLGA and PSi were the only nanoparticles able to sustain the release of GLP-1 in biological fluids when coated with CS. This characteristic was also maintained when the nanosystems came in contact with the intestinal Caco-2 and HT29-MTX cell monolayers. The CS-coated PSi nanoparticles showed the highest GLP-1 permeation across intestinal *in vitro* models. In conclusion, the PLGA+CS and PSi+CS are promising nanocarriers for the oral delivery of GLP-1.

KEYWORDS: Glucagon like peptide-1; oral delivery systems; nanoparticles; chitosan; triple co-culture; diabetes

2. Introduction

T2DM is the most prevalent metabolic disorder worldwide, making it the fourth leading cause of death in the developed countries (Russell, 2013). Currently, incretin hormones are in the pipeline for the treatment of T2DM due to their ability to potentiate insulin secretion after meal in a glucose-dependent manner. This incretin effect represents approximately 50–70% of the total insulin secreted following oral glucose administration in normoglycemics (Baggio and Drucker, 2007; Campbell and Drucker, 2013). Among all the incretin hormones, the 30 amino acids peptide GLP-1 is the most studied. Besides insulin production, GLP-1 also stimulates the neogenesis and proliferation of pancreatic β cells, which slows the progression of T2DM, and also suppresses the glucagon release (Tahrani *et al*., 2010). Moreover, its insulinotropic effect is lost when glucose concentration is below 77 mg/dL, thus overcoming hypoglycaemia which is the major side effect of the currently available therapy (Nauck *et al*., 2002; Rekha and Sharma, 2013).

Nevertheless, GLP-1 is currently administrated by parenteral routes, which are not well accepted by patients, and also do not mimic the endogenous pathway of GLP-1 secretion (Araújo *et al*., 2012; Gupta *et al*., 2013). Aiming to overcome the known disadvantages of this delivery route, attempts to deliver GLP-1 orally have been made, specially using alternative delivery systems to protect it from the harsh conditions of the gastrointestinal (GI) tract and prolong release and effect (Chae *et al*., 2008; Joseph *et al*., 2000; Nguyen *et al*., 2011). However, these studies are still very preliminary and none of these formulations have progressed towards further studies.

In this work, three different kinds of nanosystems were developed in order to deliver GLP-1 orally. These nanosystems were prepared from three different types of materials with known biocompatibility and biodegradability properties: (i) poly(lactide-co-glycolide) polymer (PLGA), (ii) lipid Witepsol E85, and (iii) porous silicon (PSi) (Bimbo *et al*., 2010; Danhier *et al*., 2012; Sarmento *et al*., 2007a; Shahbazi and Santos, 2013). These are well known biomaterials that have been extensively studied in the drug delivery field. They have the ability to help drugs to overcome the GI barriers and sustain and/or control the drug release, thus improving the therapeutic efficiency of the drug and reducing unwanted side effects. Moreover, the surface chemistry can be tailored conferring them specific properties, which is another advantage of these materials (Danhier *et al*., 2012; Fredenberg *et al*., 2011; Salonen *et al*., 2008; Sarmento *et al*., 2007a; Zhang *et al*., 2012a).

Nonetheless, the successful protection of the cargos from GI environment merely is not enough. The nanoparticles still have to face the protective mucus layer present in the intestine, which makes it difficult for the nanoparticles to approach the epithelial cells (Ensign

et al., 2012). Thus, modifying the surface of the particles with mucoadhesive biomaterial, such as CS, will promote mucus and cellular adhesion leading to the retention of the delivery systems at the site of absorption for longer periods of time, thereby increasing the oral bioavailability. Moreover, CS is also described as being able to increase the permeability of the drugs by transiently opening the cellular tight junctions (Andrade *et al*., 2011; Sarmento *et al*., 2007b; Shahbazi and Hamidi, 2013).

The aim of this work was to compare three different developed nanosystems with different properties, and select the one with most advantageous characteristics to the nanoparticles for oral delivery. Moreover, the effect of CS adsorption on the surface of these nanoparticles was also studied. For that, an extensive characterization of the nanoparticles was made, as well as the study of the GLP-1 release in biological fluids performed. The interaction studies of the nanoparticles with intestinal cell lines were also performed together with the evaluation of GLP-1 permeation across intestinal cellular monolayers.

3. Material and Methods

3.1 Materials and cell lines

GLP-1 was manufactured by United Peptides, USA. PLGA 50:50 was obtained from Purac Biomaterials, Purasorb® PDLG 5004 and Witepsol E85 from Sasol, Germany. Polyvinyl alcohol (PVA), low molecular weight CS, fluorescein isothiocyanate (FITC), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and glutaraldehyde were purchased from Sigma-Aldrich®, USA. Culture flasks and Transwell® plates were purchased from Corning Inc., USA. Dulbecco's Modified Eagle medium (DMEM), L-glutamine, non-essential amino acids, Penicillin (100 IU/mL), Streptomycin (100 mg/mL) and trypsin–EDTA were purchased from HyClone (USA). Hank's balanced salt solution (HBBS) and heat inactivated fetal bovine serum (FBS) were purchased from Life Technologies Gibco®, USA. Human colon carcinoma (Caco-2) and Raji B cell lines were obtained from American Type Culture Collection (ATCC, USA) and HT29-MTX cell line was kindly provided by Dr. T. Lesuffleur (INSERM U178, Villejuif, France).

3.2 Preparation of PLGA nanoparticles and SLNs

Different formulations of polymeric and lipidic nanoparticles were prepared through modified solvent emulsification–evaporation method, based on the water-in-oil-in-water (w/o/w) double emulsion technique (García-Fuentes *et al*., 2003; Zhang *et al*., 2006). The polymer/lipid was dissolved in heated ethyl acetate in a concentration of 100 mg/mL. Then, 100 µL of GLP-1 solution in MilliQ-water (2.5 mg/mL) was added to this solution and homogenised for 30 sec using a Vibra-Cell™ ultrasonic processor (Sonics®, Sonics and Matrials, Inc., USA). The primary emulsion (w/o) formed was then added into 4 mL of the surfactant solution, 2% of PVA in Milli-Q water, and homogenised again for 30 sec with the same amplitude. The second emulsion formed (w/o/w) was finally added to 7.5 mL of the same surfactant solution and was left under magnetic stirring at 300 rpm for at least 3 h for organic solvent evaporation.

CS solution was prepared by dissolving CS in deionized water containing 1% (v/v) acetic acid overnight under magnetic stirring with pH adjustment to 5.5, followed by filtration through a paper filter Millipore #2 and stored at 4ºC. To coat the nanoparticles with CS, the formulation was added into a solution of CS, at a ratio of 5:1 (w/w) regarding the solid content of the solutions, and left overnight under magnetic stirring, allowing surface layer deposition of the polymer on the surface of the particles.

Nanoparticles were washed three times in MilliQ-water and separated through centrifugation, using an ultra-centrifuge (Optima™ TL, Beckman Coulter, USA), at 34300 × *g* for 20 min for PLGA nanoparticles and 250000 × *g* for 150 min for SLNs. After centrifugation, nanoparticles were re-dispersed in water and stored at 4°C for further analysis.

Drug-free nanoparticles were prepared according to this procedure but using MilliQ-water instead of GLP-1 solution as internal aqueous solution. In order to visualize the nanoparticles, FITC was also loaded into the nanoparticles according to the procedure described before, but using FITC-solution instead of GLP-1 solution.

3.3 Preparation of the PSi nanoparticles

Free-standing multilayer PSi films were anodized using single crystal Si wafers ⟨100⟩ of p+-type with resistivity values of 0.01–0.02 Ωcm in hydrofluoric acid (40%)–ethanol mixture. The anodization profile consisted of successive low, high and zero current pulses applied on the Si wafer. Hydrocarbonization treatment for the multilayer films consisted of exposing the particles to a 1:1 (v/v) flow of N_2 and acetylene for 15 min at room temperature, followed by 15 min at 500 °C, and then cooling down to room temperature under N_2 flush.

The obtained thermally hydrocarbonized PSi (THCPSi) was then modified with 10-undecenoic acid for 16 h at 120 °C to obtain partially carboxylic acid terminated PSi (UnTHCPSi). Wet ball milling was used to reduce the size of the modified PSi films, while the surface oxidation was minimized by using a 5 vol-% 10-undecenoic acid - dodecane solution as the milling liquid. The final size separation and exchange of the suspension media were done by centrifugation (Bimbo *et al*., 2010; Shahbazi *et al*., 2014).

For the GLP-1 loading, a ratio of 1:5 (w/w; GLP-1:PSi) was used for GLP-1-loaded PSi nanoparticles. The GLP-1 loading was performed by dispersing the PSi nanoparticles in GLP-1 solution and stirring at room temperature for 90 min at 300 rpm. After that, the suspension was centrifuged at 27600 $\times g$ for 5 min to remove the excess free GLP-1. To coat the nanoparticles with CS through physical adsorption, the nanoparticles were first centrifuged at 27,600 $\times$ *g* for 5 min, and then mixed with a solution of CS at a ratio of 5:1 (w/w) regarding the solid content of the solutions, and left overnight under magnetic stirring at 300 rpm. For the nanoparticles coated by physical adsorption with CS, the GLP-1 loading was performed prior to the coating in order to prevent the blockage of the nanoparticles' pores by CS (Shrestha *et al*., 2014).

3.4 Nanoparticles characterization

After production, nanoparticles were characterized for their average particle size (*Z*-average), polydispersity index (PdI) and average zeta–potential by electroforethic light scattering using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd, UK). For these measurements, samples were diluted in MilliQ-water.

To characterize the morphology of the nanoparticles and confirm their size distribution, nanoparticles were observed by transmission electron microscopy (TEM, Tecnai^{TM} F12, FEI Company, USA) using an acceleration voltage of 120 kV. For sample preparation, nanoparticles were diluted in MilliQ-water, placed on copper coated grids, and contrasted with uranyl acetate. In each step, the excess of water was removed using a filter paper, and finally, grids were left at room temperature for a few minutes to dry before the imaging.

3.5 Association efficiency (AE) and loading degree (LD)

To determine the AE and LD of the developed nanosystems, the amount of GLP-1 associated to the nanoparticles was calculated. This calculation was made by the difference between the total amount of GLP-1 used to prepare the systems, and the amount of GLP-1

that remained in the aqueous phase after nanoparticles isolation by centrifugation, according to the follow equations (Li *et al*., 2013; Sarmento *et al*., 2006):

$$AE\ (\%) = \frac{Initial\ mass\ GLP{-}1 - mass\ of\ GLP{-}1\ in\ supernatant}{Total\ mass of\ GLP{-}1} \times 100$$

$$LD\ (\%) = \frac{Initial\ mass\ GLP{-}1 - mass\ of\ GLP{-}1\ in\ supernatant}{mass\ of\ nanoparticles} \times 100$$

The amount of GLP-1 was determined by HPLC (Agilent 1260, Agilent Technologies, USA), using a C_{18} column (4.6 × 150 mm, 5 µm, Zorbax Eclipse plus C18, USA). The mobile phase consisted of 0.1% trifluoroacetic acid (pH 2.0) and acetonitrile initially set at the ratio of 70:30 (v/v), which linearly changed to 60:40 (v/v) as a gradient over 5 min. Afterwards, the ratio was kept constant for 5 more minutes. The flow rate was 1.0 mL/min and the injected volume of the sample was 75 µL. The column temperature was set at room temperature and the detection wavelength at 240 nm. The total area under the curve (AUC) was used to quantify the GLP-1.

3.6 *In vitro* release studies

The GLP-1 loaded nanoparticles (corresponding to 30 µg of GLP-1) were added to 20 mL of pH 1.2 buffer (50 mM KCl) to simulate the gastric fluid. After 2 h, nanoparticles were centrifuged and introduced in a fasted state stimulated intestinal fluid (FaSSIF) (50 mM KH_2PO_4, 15 mM NaOH, 1.0 % (w/v) pancreatin; pH 6.5) for 4 h more. Aliquots of 750 µL were collected at specific time points (0.5, 1, 2, 3, 4, 5 and 6 h) during the dissolution experiments and withdrawn volume was replaced with fresh buffer, keeping the volume constant. All the collected aliquots were centrifuged at 27,600 × *g* for 20 min and the supernatant was used for HPLC analysis in order to quantify the GLP-1 released from the nanoparticles over time. All the tests were performed at 37ºC and 100 rpm under sink conditions.

3.7 Cell culturing

Caco-2 (passages #31–40) and HT29-MTX (passage #15–30) cells grew separately in culture flasks in a complete medium consisting of DMEM supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine, 1% (v/v) NEAA, and 1% (v/v) antibiotic–antimitotic mixture (final concentration of 100 U/ml Penicillin and 100 U/ml Streptomycin). Cells were sub-cultured once a week using 0.25% trypsin–EDTA (1×) to detach the cells from the flasks and seeded at a density of 0.5×10^6 cells per 75 cm^2 flask. The culture medium was replaced every other day. Cells were maintained in an incubator (BB 16 gas incubator, Heraeus Instruments GmbH) at 37ºC and 5% CO_2 and 95% relative humidity. Raji B cells (passages #26–35) were cultured in flasks with DMEM supplemented and at the same conditions as described above.

3.8 Cell viability studies

For the viability tests, 100 µL of Caco-2 and HT29-MTX cells, at 0.5×10^6 cells/mL, were seeded separately in 96-well plates and were allowed to attach for 24 h. After that, the medium was aspirated and the wells were washed twice with 100 µL of fresh HBSS-HEPES buffer (pH 7.4). After washing, 100 µL of the nanoparticle solutions corresponding to GLP-1 amounts of 1.5, 3.75, 7.5 and 15 µg/mL of GLP-1 were added to each well and the plates were incubated for a period of 3 and 12 h at 37ºC. Afterwards, the plates were equilibrated at room temperature for about 30 min and then washed twice with 100 µL of HBSS–HEPES (pH 7.4). About 50 µL of the reagent assay CellTiter-Glo® (Promega Corporation, USA) were added to 50 µL of HBSS–HEPES (pH 7.4) in each well; negative (HBSS buffer) and positive (1% Triton X-100) control wells were also used and treated similarly as described above. The plate was mixed for 10 min on an orbital shaker at room temperature, protected from the light. Finally, the luminescence was measured using Varioskan Flash plate reader (Thermo Fisher Scientific Inc., USA). The assay is based on the amount of ATP produced by metabolically active cells, which is directly proportional to the number of living cells present in the culture (Bimbo *et al*., 2011). All data sets were compared with a negative control of HBSS-HEPES (pH 7.4), considered as 100% viability.

3.9 Cell–nanoparticles interactions

To verify the interactions between cells and nanoparticles, Caco-2:HT29-MTX co-cultures in the proportion of 90:10 were seeded in Lab-Tek 8-chamber slides (Thermo Fisher Scientific Inc., USA) and allowed to attach for 24 h. Afterwards, cells were washed twice with fresh HBSS–HEPES buffer (pH 7.4). Then, FITC-loaded nanoparticles were added to cells

and incubated at 37°C for 3 h. After incubation, cells were washed twice with fresh HBSS–HEPES buffer (pH 7.4). Then, the plasma membrane was stained by adding 200 µL of CellMask™ Orange (Invitrogen, USA) and incubated for 3 min at 37°C. The excess of staining solution was washed twice with fresh HBSS–HEPES buffer and cells were fixed using 2.5% glutaraldehyde for 20 min. The localization of the FITC-loaded nanoparticles was then observed using a Leica SP5 confocal microscope (Leica Microsystems, Germany).

3.10 Permeability experiments

For the permeability experiments, 7×10^4 cells/cm^2 of Caco-2 and HT29-MTX cells in a ratio of 90:10 were seeded in 12-Transwell® cell culture inserts and were allowed to grow and differentiate for 14 days with medium replacement every other day. Then, 1.0×10^5 Raji B cells were added to the basolateral chamber for 7 days more, in order to induce the phenotype change of Caco-2 cells into M cells and to obtain a triple co-culture model (Araújo and Sarmento, 2013). The permeability experiments across the cell monolayers were performed in the apical-to-basolateral direction in HBSS–HEPES at pH 6.5 (apical compartment) and pH 7.4 (basolateral compartment) at 37ºC using an orbital shaker (100 rpm). After removing the cell culture medium, 0.5 mL of pure GLP-1 or nanoparticles in the concentration of 2 µg/mL were pipetted into the apical side of the inserts. At different time points (0.5, 1, 2 and 3 h), 0.75 mL samples were taken from the basolateral side of the inserts and the same volume of fresh HBSS–HEPES (pH 7.4) buffer was added to replace withdrawn volume. Sample concentrations were quantified by EIA GLP-1 Kit (Sigma-Aldrich®, USA) according to manufacturer's instructions. The integrity of the cell monolayers was checked before and after the permeability experiment by measuring the trans epithelial electric resistance (TEER) using Millicell®-Electrical Resistance System (Millipore, USA).

3.11 Morphological characterization of co-culture monolayers

After the drug permeation studies, the nanoparticles suspension was carefully removed and cells were fixed with 2.5% glutaraldehyde for 20 min at room temperature. The wells were then washed twice with sodium cacodylate buffer (NaCac) for 3 min. Afterwards, cells were post-fixed with 1% osmium tetroxide in 0.1 M NaCac buffer (pH 7.4) and then dehydrated and embedded in epoxy resin. Ultrathin sections (60 nm) were cut perpendicular to the insert, post-stained with uranyl acetate and examined with TEM using an acceleration voltage of 120 kV.

3.12 Statistical analysis

All the experiments were performed in triplicate and are represented as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) with Bonferroni post hoc-test (GraphPadPrism, GraphPad software Inc., CA, USA) was used to analyse the data. The level of significance was set at probabilities of $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

4. Results and Discussion

4.1 Characterization of the nanoparticles

Nanoparticles were developed and characterized on the basis of their particle size, PdI and zeta-potential, as shown in **Table 4**. All of the uncoated nanoparticles presented average sizes of approximately 200 nm with low PdI values (≤ 0.12), suggesting the monodispersity of the nanosystems. TEM images supported these results in terms of mean size and their homogenous distribution (**Figure 12**). From the TEM images, it can also be observed that SLN and PLGA nanoparticles have spherical shape, in contrast with the irregular shape of UnTHCPSi nanoparticles. The shape of nanoparticles has also been described as having an impact on their interaction at cellular level (Verma and Stellacci, 2010), wherein round-shaped nanoparticles are taken up more easily than irregular ones with sharp edges (Caldorera-Moore *et al*., 2010). Regarding the surface charge, it was observed that all of the uncoated nanoparticles are negatively charged due to the nature of the biomaterials used. However, nanoparticles with negative charge would lead to electrostatic repulsion with the negatively charged mucus layer on the intestinal wall (−50 mV), thereby inhibiting the nanoparticles to move closer to the intestinal epithelial wall (Zhang *et al*., 2012). Thus, to improve the interaction of the nanosystems with the intestinal epithelia, the nanoparticles were then coated with CS, a positively charged mucoadhesive polymer.

The inheritance of positive charge from CS to the nanoparticles enhances the interactions with negatively charged mucus and cells, which can lead to improvement in the permeability and/or cellular internalization of the cells (Wang *et al*., 2013; Zhang *et al*., 2012). Moreover, CS is described as being an efficient intestinal absorption enhancer since it is able to transiently open tight junctions between epithelial cells (Andrade *et al*., 2011; Chen *et al*., 2013). The successful surface modification of the nanoparticles with CS was observed by the significant change in the zeta-potential and by the significant increase in the particle size, as shown in **Table 4**. However, the shape of the nanoparticles did not change as seen in **Figure 13**. These changes are in accordance with other studies, in which modification of nanoparticles with CS were also performed (Wang *et al*., 2013; Zhang *et al*., 2012).

Table 4. Size, PdI, ζ-potential, association efficiency (AE) and loading degree (LD) of the different nanoparticles. Results are presented as mean ± SD ($n \geq 3$). The values of uncoated nanoparticles were compared with their corresponding CS-coated nanoparticles, $^*p < 0.05$.

	Size (nm)	PDI	ζ-potential (mv)	AE (%)	LD (%)
PLGA	182.9 ± 5.3	0.08 ± 0.02	−20.3 ± 1.0	67.0 ± 10.9	0.17 ± 0.03
PLGA + CS	198.4 ± 5.7*	0.11 ± 0.01	+17.5 ± 1.2*	60.0 ± 0.8	0.15 ± 0.01
SLN	217.4 ± 2.0	0.11 ± 0.02	−7.5 ± 0.3	73.3 ± 0.6	0.18 ± 0.01
SLN + CS	223.6 ± 1.1*	0.11 ± 0.01	+13.4 ± 0.3*	57.0 ± 5.6*	0.14 ± 0.01
UnTHCPSi	196.8 ± 6.0	0.12 ± 0.02	−30.1 ± 2.8	85.0 ± 0.6	17.00 ± 0.05
UnTHCPSi + CS	363.0 ± 8.0*	0.23 ± 0.02	+19.2 ± 0.4*	85.0 ± 0.5	14.16 ± 0.04

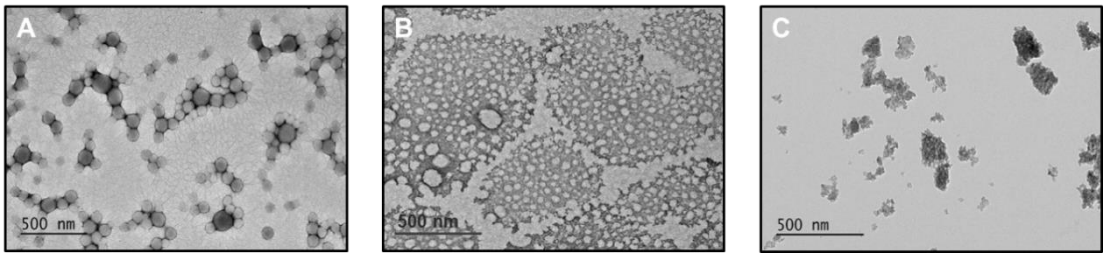

Figure 12. TEM images of (**A**) PLGA nanoparticles; (**B**) SLN nanoparticles and (**C**) UnTHCPSi nanoparticles.

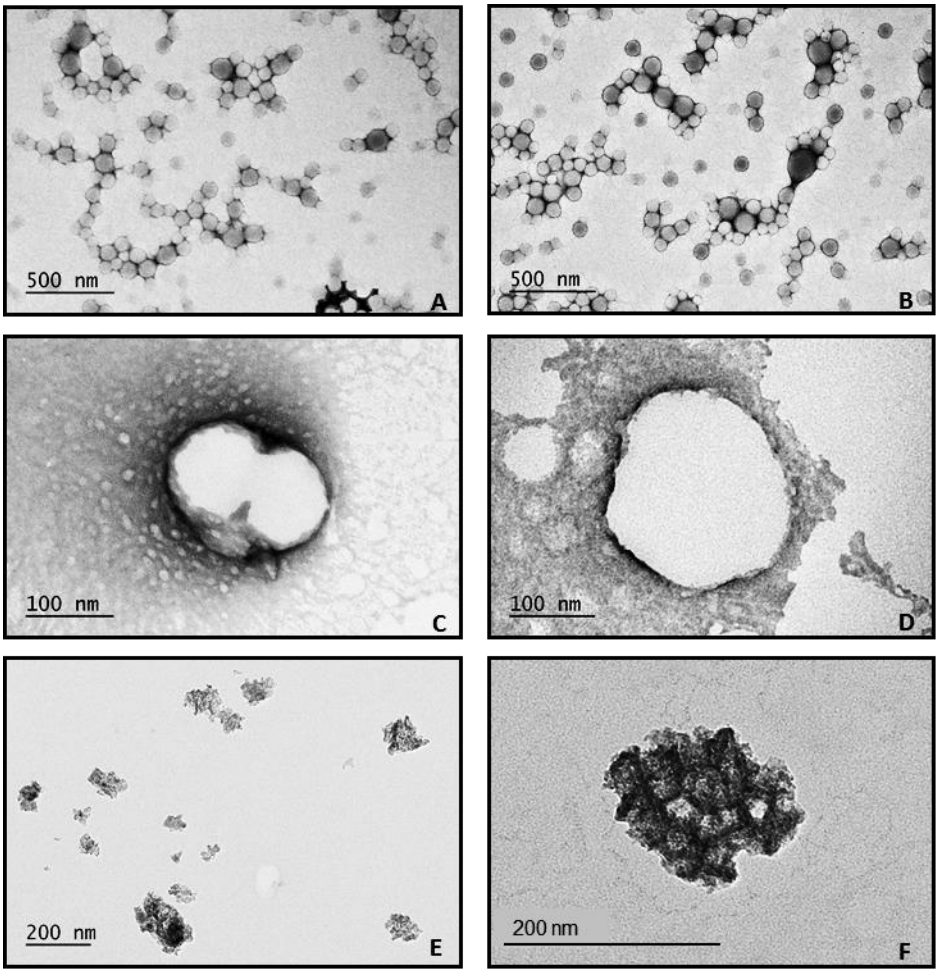

Figure 13. TEM images of (**A**) PLGA nanoparticles and (**B**) PLGA+CS nanoparticles; (**C**) SLN nanoparticles and (**D**) SLN+CS nanoparticles and (**E**) UnTHCPSi nanoparticles and (**F**) UnTHCPSi+CS nanoparticles.

4.2 Association efficiency (AE) and loading degree (LD)

UnTHCPSi nanoparticles, coated and uncoated with CS, presented the highest AE with 85 ± 1% of the initial peptide associated to the nanoparticles **(Table 4)**. For PLGA nanoparticles, the AE of GLP-1 was 67 ± 11% which was in the same range of values as CS coating (PLGA+CS) with 60 ± 1% of AE ($p > 0.05$). In turn, SLN had significantly higher AE than CS-coated SLN nanoparticles (SLN+CS), with values of 73 ± 1% and 57 ± 6%, respectively. These reductions in AE for the CS-coated nanoparticles may be due to an additional step of mixing the nanoparticles with the CS overnight which could have led to some release of the drug (Wang *et al*., 2013). Taking into account that GLP-1 is a hydrophilic

peptide, the AE obtained with these systems seemed to be higher than the usual values obtained for other hydrophilic peptides such as insulin (Emami *et al*., 2009; Fonte *et al*., 2011; Sarmento *et al*., 2007a; Zhang *et al*., 2012). It could be explained by the production method used and the use of ethyl acetate as organic solvent, which is known to enhance the rate of encapsulation of hydrophilic molecules (Cohen-Sela *et al*., 2009).

Regarding the LD, UnTHCPSi nanoparticles also had the highest LD of 17 %, which is 100 times higher than that of PLGA and SLN nanoparticles. Regarding the CS-coated nanoparticles it was observed that the LD values were slightly lower with no significant changes when compared with the uncoated nanoparticles, except in UnTHCPSi where it remained the same (**Table 4**).

Such differences in the AE and LD of PLGA and SLN in comparison with UnTHCPSi are explained with the methods used in their production. With the UnTHCPSi nanoparticles, the payload will be retained in the pores of the nanoparticles, generally by physical adsorption. The electrostatic interactions towards the payload will be the main factors contributing for its incorporation and retention in the nanoparticles (Kovalainen *et al*., 2012; Liu *et al*., 2013). However, for the PLGA and SLN nanosystems, the payload needs to be entrapped into the nanoparticles. During the production of these nanoparticles, several different stages of the double emulsion method are involved, which increases the probability of loss of the peptide (Almeida and Souto, 2007; Liu *et al*., 2013).

4.3 Cell viability studies

The *in vitro* cell viability experiments were performed using two different intestinal cell lines, Caco-2 and HT29-MTX. As Caco-2 cells resemble enterocytes that represent approximately 90% of the total epithelial cells of the intestine and HT29-MTX mimic the goblet cells that are approximately 10% of intestinal cells (Araújo and Sarmento, 2013), these cell lines are suitable *in vitro* models to mimic the intestinal epithelia (Antunes *et al*., 2013; Araújo and Sarmento, 2013; Sarmento *et al*., 2012). Cells viability was measured after exposure of the nanoparticles to the cells at different concentrations and incubation periods. The aim of using different concentrations is to understand if there is any concentration dependent toxicity and to know the lowest safe concentration that can be administrated. Two different incubation periods, 3 and 12 h, were tested because they represent the average and maximum transit time in the intestinal tract, respectively (Shah *et al*., 2010). The results are presented in **Figure 14.**

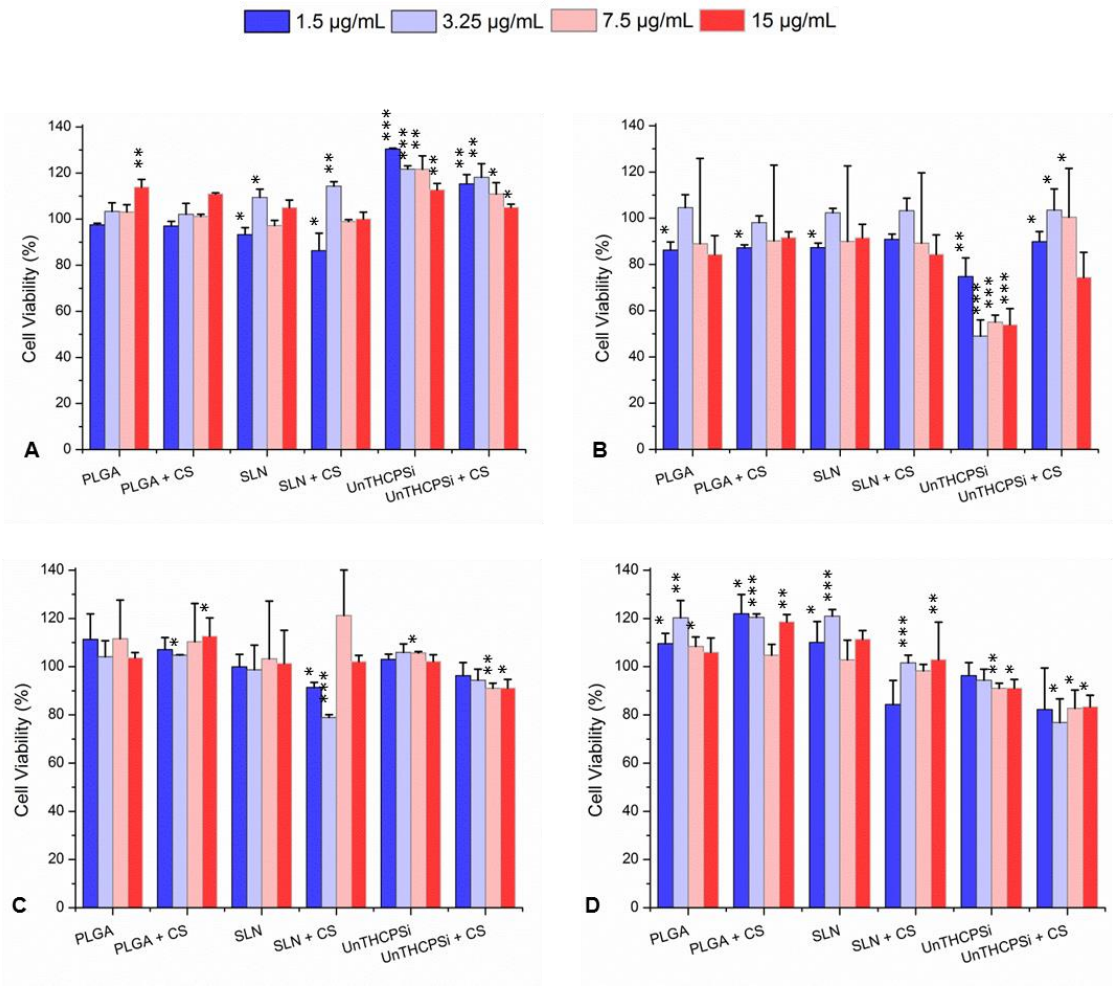

Figure 14. Cell viability of the intestinal cells after exposure to PLGA, SLN and UnTHCPSi nanoparticles (uncoated and coated with CS) assessed by the CellTiter-Glo® luminescence assay. The viability of (A and B) Caco-2 and (C and D) HT29-MTX cells after (A and C) 3 h and (B and D) 12 h incubation with different nanoparticles concentrations at 37ºC. All data sets were compared to the negative control HBSS buffer. Error bars represent mean ± SD ($n \geq 3$); *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

For Caco-2 cells, it can be observed that the viability of the cells was about 100% for all concentrations tested after 3 h of exposure to the nanoparticles (**Figure 14A**). After 12 h, the cell viability decreased in general for all formulations; nevertheless, all of the cells presented viability above 80%, with the exception of UnTHCPSi nanoparticles, which presented lower viability after 12 h (**Figure 14B**). Regarding HT29-MTX cells, after 3 h incubation, the cell viability for all the formulations was very similar to the control, approximately 100%, with the exception of SLN+CS (**Figure 14C**). After 12 h, no significant differences to the control were found for all samples (**Figure 14D**). With HT29-MTX cells, the

nanoparticles showed less toxicity compared to Caco-2 cells at the same time-point. This may be explained by the fact that the HT29-MTX cells are mucus-producing cells, and the mucus layer protects the cells from interacting strongly with the nanoparticles, minimizing the cytotoxicity associated with them (Araújo and Sarmento, 2013; Sarmento *et al*., 2012).

4.4 *In vitro* release studies

The *in vitro* release tests intended to predict the release profiles of GLP-1 in conditions similar to GI tract (stomach and small intestine). Firstly, the nanoparticles were added to pH 1.2 buffer, which mimics the gastric environment. Then, after 2 h, the release medium was changed to FaSSIF (pH 6.5), which simulates the transit of the nanoparticles to the small intestine. The release profiles of GLP-1 from the uncoated and CS-coated nanoparticles are shown in **Figures 15A** and **15B**, respectively.

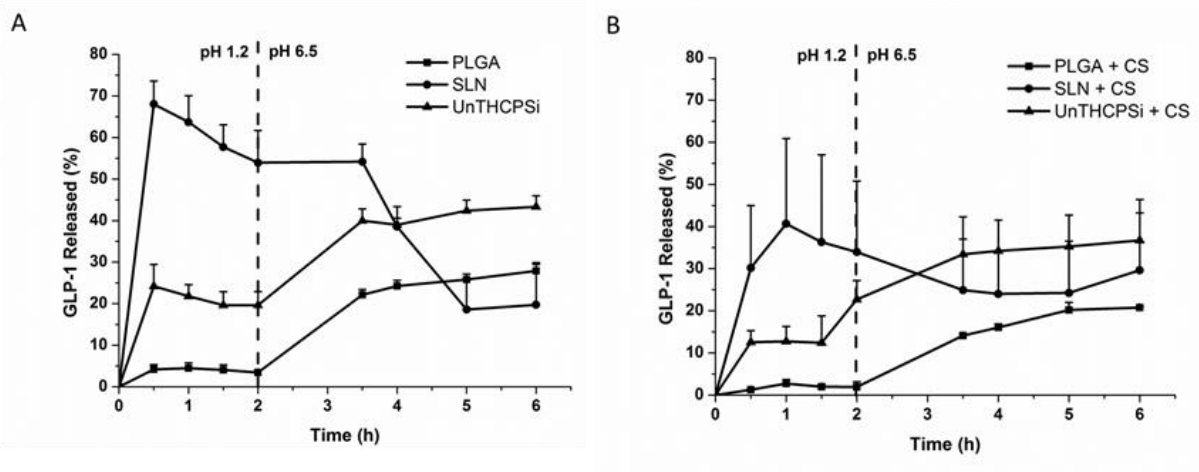

Figure 15. Release profiles of GLP-1 from (**A**) PLGA, SLN and UnTHCPSi nanoparticles and from (**B**) PLGA+CS, SLN+CS and UnTHCPSi+CS nanoparticles. The percentages of peptide released from uncoated nanoparticles were compared with their corresponding CS-coated nanoparticles at the same time points and compared with the other nanoparticles. All experiments were conducted at pH 1.2 in the first 2 h and in FaSSIF (pH 6.5) for 4 h more at 37 °C and 100 rpm. Errors bars represent the mean ± SD (n = 3).

PLGA nanoparticles did not release significant amounts of GLP-1 at pH 1.2 for 2 h. At such low pH, the charge of GLP-1 (isoelectric point pI = 5.4) is highly positive, and thus, the interactions with the negatively charged PLGA will be very strong resulting in lower percentages of the peptide released (Huotari *et al*., 2013; Li *et al*., 2008; Martins *et al*., 2007; Sarmento *et al*., 2006; Sarmento *et al*., 2007b). Moreover, the PLGA nanoparticles preserved

their physical integrity avoiding the nanoparticle erosion, which also contributed to the reduced GLP-1 release (Li *et al*., 2008; Martins *et al*., 2007; Sarmento *et al*., 2006). When added to the FaSSIF, a significant release of 22 ± 1.3 % was observed, reaching 28 ± 1.6% of GLP-1 release after 6 h. This could be attributed to the increase in the pH, which results in a change of the charge of the peptide, thereby causing a reversion in the interaction with the nanoparticles and leading to the release of the peptide (Martins *et al*., 2007; Sarmento *et al*., 2006). The biphasic release pattern of drugs from the PLGA nanoparticles has been frequently reported in the literature (Buske *et al*., 2012; Emami *et al*., 2009; Fredenberg *et al*., 2011; Giteau *et al*., 2008). Regarding the PLGA+CS nanoparticles, a similar GLP-1 release profile was observed as with the PLGA nanoparticles but in a more sustained pattern. The absence of release at pH 1.2 was followed with a considerable release of 14.1 ± 0.4% in the FaSSIF that reached 21% after 6 h. The decreased release of PLGA+CS in comparison with uncoated PLGA nanoparticles in FaSSIF can be explained by the interaction of the non-encapsulated GLP-1. This GLP-1–CS interactions probably led to a better release control of GLP-1 from the nanoparticles (Zhang *et al*., 2012). The results obtained are in agreement with other reports, in which CS has been described as being able to decrease the burst release effect of encapsulated drugs (Chakravarthi and Robinson, 2011; Gupta *et al*., 2013).
For UnTHCPSi and UnTHCPSi+CS nanoparticles, burst releases of 24.2 ± 5.2% and 11.8 ± 6.3% were, respectively, observed at pH 1.2 in the first 30 min followed by a constant release plateau. In FaSSIF, a rapid release was again observed for both nanoparticles, followed by a sustained GLP-1 release for the next 4 h. At 6 h, the total percentage of GLP-1 released from the UnTHCPSi and UnTHCPSi+CS were 43.3 ± 2.7% and 35.3 ± 6.5%, respectively. Once again, the CS-coated UnTHCPSi nanoparticles provided less release than the uncoated ones, as was also seen for the PLGA nanoparticles. These results confirm that the coating of the nanoparticles with CS sustained the release of GLP-1.

For the SLN nanoparticles, a significant burst release of 68.0 ± 5.5% in the first 30 min was observed at pH 1.2. After this time-point, the percentage of GLP-1 was decreased over time due to its degradation, as observed by HPLC (data not shown). Even in FaSSIF, the percentage of GLP-1 did not increase, being the GLP-1 released residual when compared to the degraded GLP-1. The burst release followed by a sustained release in this kind of systems is common since hydrophilic peptides tend to accumulate at the o/w interface during preparation, thus remaining at the surface of the nanocarrier and in this case, the nanoparticles were not able to control the release of the peptides (Almeida and Souto, 2007). Also of relevance is the fact that SLN were not as much negatively charged as the other nanoparticles tested, thus the interaction with the positively charged GLP-1 was not as strong as observed for the other nanosystems, resulting in a higher percentage of released GLP-1. In contrast, SLN+CS nanoparticles promoted a more sustained release profile, with around

40% of the peptide released in the first hour, after which the GLP-1 started to decrease. Previous studies showed that under the same conditions, uncoated SLN nanoparticles suffered 80% more degradation at 4 h than the coated nanoparticles (Almeida and Souto, 2007; Garcia-Fuentes *et al*., 2005). Thus, CS decreases the burst release effect of the encapsulated peptides after administration and also increases the stability of nanoparticles and macromolecules (Chakravarthi and Robinson, 2011; García-Fuentes *et al*., 2003; Gupta *et al*., 2013).

Overall, the PLGA is the system which could efficiently retain the GLP-1 from the harsh environment of the simulated conditions of the stomach without peptide release at pH 1.2, with sustained GLP-1 release thereafter. Despite the release at pH 1.2, the UnTHCPSi systems had a very similar behaviour to PLGA systems, as the total percentage of GLP-1 available in FaSSIF at the end of 6 h was in the same range (15-20%). Regarding the SLN nanoparticles, they do not retain the peptide at low pH, causing a burst release (more than 50% of the loaded peptide) in the first 30 min at pH 1.2. Although CS coating more efficiently controlled the release from SLN, it still showed a significantly higher GLP-1 release at pH 1.2 in comparison to the PLGA+CS and UnTHCPSi+CS nanoparticles. The differences between the uncoated and coated nanoparticles suggested that CS improve the protection of the GLP-1 and its release, providing a controlled drug delivery system.

4.5 Interaction of the nanoparticles with Caco-2:HT29-MTX co-culture cells

As stated before, Caco-2 and HT29-MTX cells represent the two most abundant cells in the intestinal epithelia. Thus, Caco-2:HT29-MTX co culture in a 90:10 proportion is a reliable model to predict the behaviour of the nanoparticles when in close contact with the intestinal mucosa (Araújo and Sarmento, 2013). The interaction between the nanoparticles and Caco-2:HT29-MTX co-culture cells were observed with confocal microscopy. In order to visualize the nanoparticles, they were loaded with FITC, rendering them the green colour for easy detection. The cell membranes were stained with red using CellMask™ Orange.

As it can be seen in **Figure 16**, no interaction was seen between the cells and the uncoated nanoparticles. Regarding the CS-coated nanoparticles, it was observed that even after multiple washes, the nanoparticles were still present in contact with the cells.

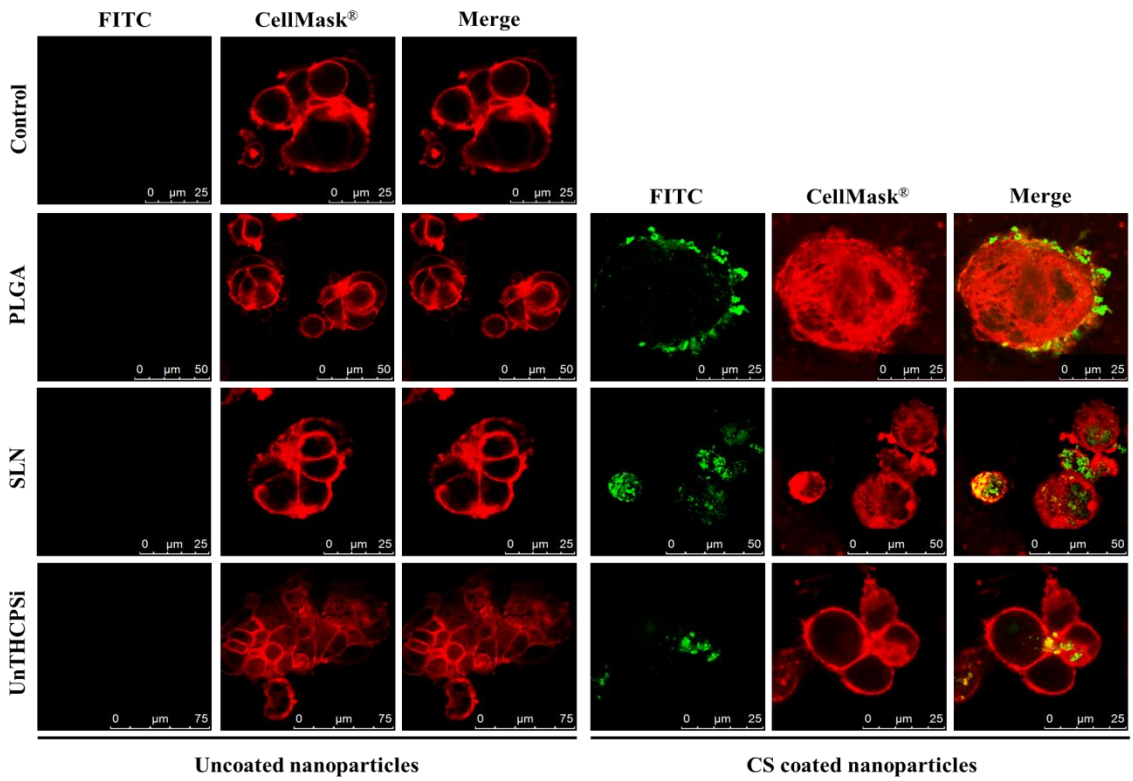

Figure 16. Confocal microscopy images of Caco-2:HT29-MTX (90:10) co-culture treated with different FITC-loaded nanoparticles after incubation for 3 h at 37°C. Red: cell membranes stained with CellMask®: Orange; green: FITC.

In fact, there were some places where it was possible to observe the yellow colour, resulting from the overlap of the green and red labelling, revealing a very close contact with nanoparticles co-localized with the cell membranes. This result was expected, due to the presence of the mucoadhesive CS on the surface of the nanoparticles which potentiates stronger electrostatic interactions with the mucus present in the cellular co-culture and also because it is positively charged, gaining much more affinity to the cells membrane (Andrade *et al*., 2011; Sarmento *et al*., 2007b).

4.6 Cell co-culture monolayers and permeability studies

To study the permeability of GLP-1 loaded into the three different CS-coated nanosystems, a triple co-culture model previously developed by our group was used. This model comprises the most important features present in the intestine, namely the presence of enterocytes (Caco-2 cells), the mucus producing goblet cells (HT29-MTX) in physiological proportions (90:10) and the induction of M-cells, as described elsewhere (Araújo and

Sarmento, 2013). M-cells may have an important role in intestinal absorption of drugs since they are specialized for antigen and microorganisms uptake, providing a possible gateway for the absorption of proteins, as well as for nanoparticles (des Rieux *et al*., 2005). Together with Caco-2 and HT29-MTX cells, they form a monolayer, where cells are joined to each other by tight junctions, mimicking the intestinal epithelia (Sarmento *et al*., 2012).

The permeability profiles of GLP-1 alone and from different nanosystems are shown in **Figure 17**. The highest GLP-1 permeation, when free in solution, may be explained with the high amount of peptide available during the time of the test, whereas a slower GLP-1 released from the nanoparticles was observed. Moreover, since these experiments were performed in HBSS-HEPES buffer to minimize the damage of the cellular monolayers, and the buffer did not present any surfactant as in FaSSIF, the GLP-1 release from the nanoparticles may have more sustained pattern than could be predicted, thereby explaining the higher permeability of GLP-1 in free solution. Regarding the nanoparticles, the UnTHCPSi+CS nanoparticles had the highest amount of GLP-1 permeated across the intestinal cell monolayers followed by SLN+CS nanoparticles ($p > 0.05$) and then by the PLGA+CS nanoparticles ($p < 0.05$). These results are in accordance with the data obtained for the *in vitro* release tests that showed that PLGA+CS nanoparticles sustained the release of GLP-1 longer than the other nanoparticles. The results obtained for SLN+CS nanoparticles also showed a sustained GLP-1 release at pH 6.5. However, in this case, the GLP-1 release was not observed in the *in vitro* release studies, mostly because of the degradation of the GLP-1 released at pH 1.2, as previously discussed. The values of the transepithelial electrical resistance (TEER), measured before and after the experiment were maintained (results not shown), demonstrating the integrity of the co-culture cell monolayer (Araújo and Sarmento, 2013). The presence of mucus is one of the most important characteristics of the cell model to predict the absorption of drugs *in vivo*, since the mucus present in the intestinal epithelia can significantly limit the effectiveness of the drug delivered by the nanosystems (Araújo and Sarmento, 2013; Ensign *et al*., 2012; Zhang *et al*., 2012). However, the retention of the coated nanoparticles on the cellular mucus layer, due to CS mucoadhesive properties, may improve the GLP-1 absorption, and thus, can also improve its bioavailability (Andrade *et al*., 2011).

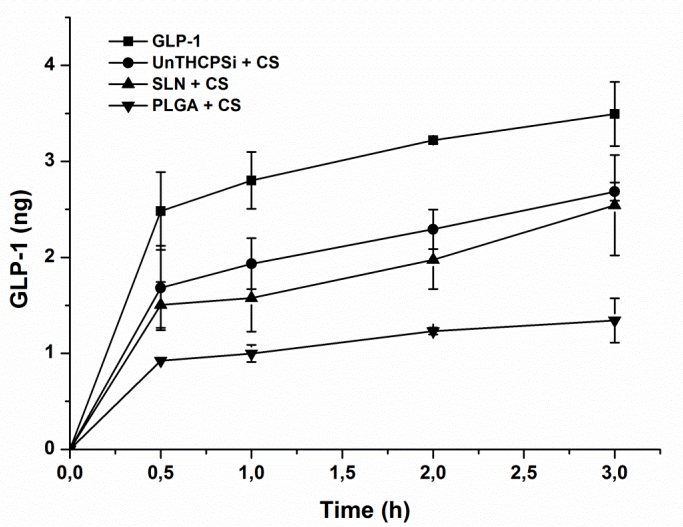

Figure 17. *In vitro* cumulative permeability profiles of GLP-1 loaded CS-coated nanoparticles across Caco-2:HT29-TX:Raji B co-culture monolayers. All experiments were conducted from the apical (pH 6.5) to basolateral direction (pH 7.4) in HBSS at 37°C. Error bars represent mean ± SD (*n* = 3).

It is possible to observe that all of the CS-coated nanoparticles were in close contact with the cells. In fact, some of the UnTHCPSi+CS nanoparticles were found inside of the cells as indicated by the arrow in **Figure 18** and shown in **Figure 19**. CS coating of the nanoparticles improved some of their characteristics, especially the positive charge, which led to cellular internalization (Huhn *et al*., 2013; Lee *et al*., 2010). These results are in agreement with other reports in the literature that showed that positive surface charge can interact with the negative charge moieties of the cell membrane, and lead to internalization (Fuller *et al*., 2008; Shahbazi *et al*., 2014)

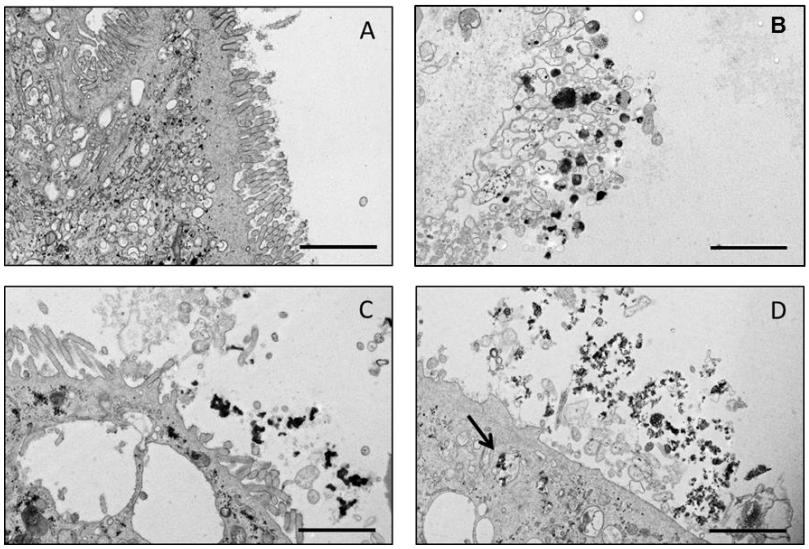

Figure 18. TEM images of flat embedded ultrathin sections after permeability showing (**A**) control cells (**B**) PLGA+CS nanoparticles, (**C**) SLN+CS nanoparticles and (**D**) UnTHCPSi+CS nanoparticles.

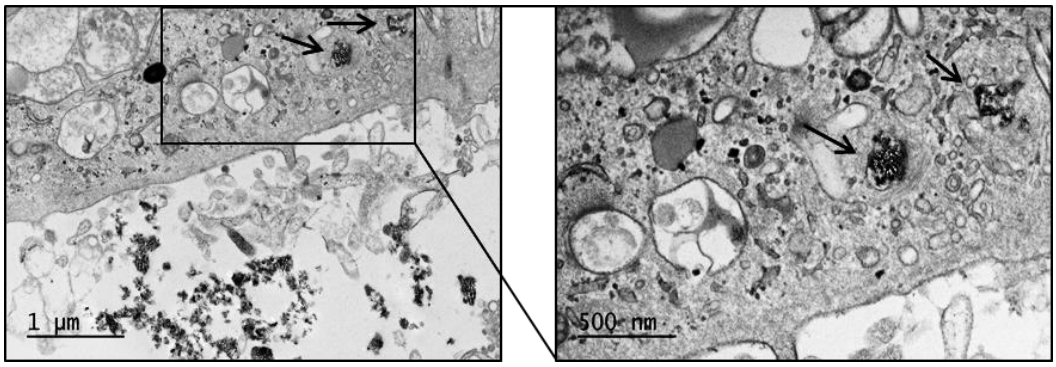

Figure 19. TEM images of flat embedded ultrathin sections after permeability showing UnTHCPSi+CS nanoparticles with some nanoparticles internalized into the cells (arrows).

5. Conclusions

In this study, three different nanosystems were compared with regards to their ability to sustain the release of GLP-1 through the harsh environment of the simulated GI tract conditions. In addition, the interactions between the developed nanosystems and the intestinal cell lines were also evaluated. CS-coated nanosystems presented better results than their corresponding uncoated nanoparticles in terms of sustained GLP-1 released and improved interaction with the intestinal cells. Among of the CS-coated nanoparticles, PLGA+CS and UnTHCPSi+CS presented the best characteristics for the oral delivery of GLP-1. PLGA+CS showed almost no release of GLP-1 at pH 1.2 and a controlled GLP-1 release with increasing pH over time. This was corroborated by the permeability across *in vitro* intestinal Caco-2/HT29-MTX/Raji B monolayers, where the amount of GLP-1 permeated was lower compared to the other nanoparticles. UnTHCPSi+CS showed the highest association efficiency and loading degree and due to its release profile, the amount of GLP-1 permeated across the cellular monolayer was the highest. Overall, our results showed that PLGA+CS and UnTHCPSi+CS are promising nanocarriers towards the oral delivery of GLP-1.

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CHAPTER IV

Microfluidic assembly of a multifunctional tailorable composite system designed for site specific combined oral delivery of peptide-drugs

This chapter was based in the following published paper:

- **Araújo, F.,** Shrestha, N., Shahbazi, M. A., Liu, D., Herranz-Blanco, B., Mäkilä, E. M., Salonen, J. J., Hirvonen, J. T., Granja, P. L., Sarmento, B. and Santos, H. A. 2015. Microfluidic assembly of a multifunctional tailorable composite system designed for site specific combined oral delivery of peptide drugs. ACS Nano. 9(8): 8291-302.

1. Abstract

Multifunctional tailorable composite systems, specifically designed for oral dual-delivery of a peptide (GLP-1) and an enzymatic inhibitor (DPP4 enzyme inhibitor (iDPP4)), were assemble through the microfluidics technique. Both drugs were co-loaded into these systems for a synergistic therapeutic effect. The systems were composed of CS and cell-penetrating peptide (CPP) modified PLGA and PSi nanoparticles as nanomatrices, further encapsulated in the enteric hydroxypropylmethylcellulose acetylsuccinate (HPMC-AS) polymer. The developed multifunctional systems were pH-sensitive, inherited by the enteric polymer, enabling the release of the nanoparticles only in the simulated intestinal conditions. Moreover, the encapsulation into this polymer prevented the degradation of the nanoparticles' modifications. These nanoparticles showed strong and higher interactions with the intestinal cells in comparison with the non-modified ones. The presence of iDPP4 enhanced the peptide permeability across intestinal cell monolayers. Overall, this is a promising platform to simultaneously deliver two drugs from a single formulation. Through this approach peptides are expected to increase their bioavailability and efficiency *in vivo* not only by their specific release at the intestinal level but also by the reduced enzyme activity. The use of this platform, specifically in combination with these two antidiabetic drugs, has clinical potential for the therapy of T2DM.

KEYWORDS: Chitosan; dual-delivery; microfluidics; PLGA; porous silicon.

2. Introduction

Nanoparticles composed of biocompatible and biodegradable materials have been claimed as promising candidates towards the oral administration of peptides (Gupta *et al*., 2013; Patel *et al*., 2014). These nanoparticles enhance the oral bioavailability of peptides and control their release, as well as provide a preserved environment for the encapsulated drugs (Araújo *et al*., 2014; Gundogdu and Yurdasiper, 2014; Morishita and Peppas, 2006).

Among the mostly studied materials, PLGA and PSi have attracted a lot of attention becoming the most desirable materials in the drug delivery field for the administration of macromolecules (Araújo *et al*., 2014; Santos *et al*., 2014). In one hand, PLGA features can be modulated by the ratio between the monomers which constitute the polymer, exhibiting a wide range of erosion times, favourable degradation characteristics and tuneable mechanical properties. It can be used to encapsulate numerous drugs with different physicochemical properties and also to sustain their release (Danhier *et al*., 2012; Wang *et al*., 2013). Moreover, it has extensive clinical applications, being already approved by the FDA and the EMA for parenteral administration (Danhier *et al*., 2012). On the other hand PSi nanoparticles have large surface area, pore volumes with adjustable diameters (2-50 nm), as well as higher drug loading capacity compared to the majority of the other materials (Liu *et al*., 2013a; Liu *et al*., 2014; Shahbazi *et al*., 2012). Furthermore, the drug loading is usually a simple process where the drug is retained inside the mesopores by physical adsorption or electrostatic interactions (Liu *et al*., 2013a). Nevertheless, the oral delivery of PLGA and PSi nanoparticles is not in such advanced state. The negative surface charge of the nanoparticles tends to limit their interaction with the negatively charged cell's surface, which is further intensified by the rapid turnover of the mucus and the intestinal cells (Araújo *et al*., 2014).

To tackle the limitations of these oral drug nanocarriers, the nanoparticles can be tailored with other materials to yield a variety of physical properties to overcome the main intestinal barriers, such as the mucus layer, epithelium and enzymatic degradation (Guo and Gemeinhart, 2008; Pereira *et al*., 2014). CS and cell penetrating peptides (CPP) are polycationic molecules extensively used in the drug delivery field. CS is a biopolymer that has been mainly used to modify nanoparticles due to its mucoadhesive characteristics and its properties as an intestinal permeability enhancer by transiently open the tight junctions existing between the epithelial cells (São Pedro *et al*., 2009; Sarmento and das Neves, 2012; Sosnik *et al*., 2014). CPP were originally considered as a "Trojan horse" because of their ability of entering cells without causing damage or eliciting a cellular response (Palm-Apergi *et al*., 2009; Shi *et al*., 2014), increasing the transcellular transport (Wang *et al*., 2014). Yet, a major challenge still remains for the oral delivery of peptides in order to overcome the harsh conditions of the stomach. In this regard, pH-sensitive polymers have been frequently

employed in the drug formulation and/or coating of nano- and microparticles to protect them from the very acidic gastric pH (Liu *et al*., 2014; Zhang *et al*., 2014).

In this study, multifunctional systems were developed that can simultaneously load peptides and enzymatic inhibitors in a single carrier with the aim to resist the conditions of the stomach, to enhance the nanoparticles' interactions with the intestinal mucus and epithelium, and to protect the peptides from enzymatic degradation after the release. An antidiabetic peptide, GLP-1, is in the pipeline for the T2DM therapy, and was used here as a model peptide. Due to its poor intestinal permeability, GLP-1 needs to be administrated by parenteral route, resulting in poor patient compliance (Pawar *et al*., 2014; Wang *et al*., 2015). Moreover, the success of the GLP-1 therapy is hindered by its rapid degradation (< 2 min) by the dipeptidyl peptidase 4 (DPP4) enzyme produced in the intestine (Janardhan and Sastry, 2014). Therefore, in order to achieve an efficient release and permeability across the intestinal epithelium of active GLP-1, the antidiabetic peptide was loaded into different nanoparticles composed of PLGA and PSi biomaterials. The PLGA and PSi nanoparticles were further modified with the mucoadhesive polymer CS and with an oligoarginine CPP to increase the permeability of nanoparticles across the intestinal cells. Afterwards, the nanoparticulate systems were encapsulated within an enteric polymer hydroxypropylmethylcellulose acetylsuccinate (HPMC-AS) loaded with the DPP4 inhibitor (iDPP4), using the microfluidic technique (**Figure 20**). To the best of our knowledge, this is the first time that the combination of these two drugs was formulated in a single delivery system. This innovative approach produced monodisperse and uniform particulate structures with the desired composition (Liu *et al*., 2014; Zhang *et al*., 2014). In comparison to the conventional preparation methods with microfluidics, by tuning the flow rates of the immiscible fluids, emulsions are formed with an exquisite degree of control and rather high encapsulation efficiency (Duncanson *et al*., 2012; Liu *et al*., 2013b). The resultant multifunctional systems were then characterized for size, morphology, pH-responsiveness, drug release, and synergistic antidiabetic peptide GLP-1 permeability across a triple intestinal cell co-culture model (Araújo and Sarmento, 2013) in the presence of the enzymatic iDPP4. The inhibition capacity of the DPP4 enzyme activity was also evaluated.

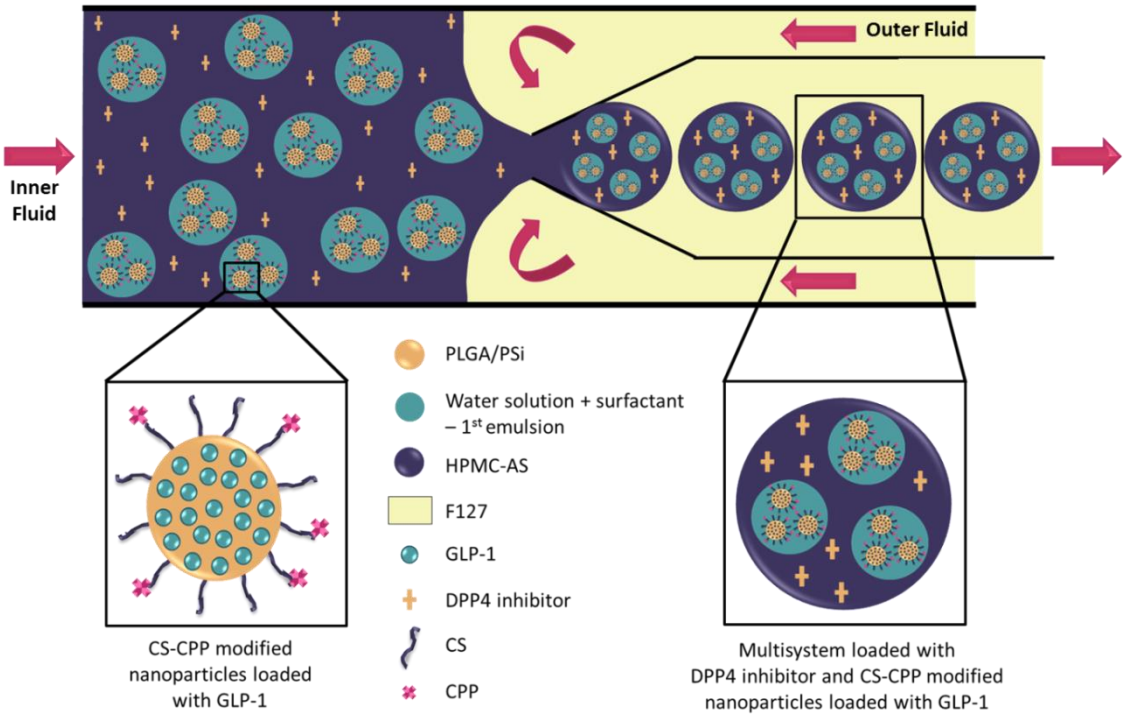

Figure 20. Schematic representation of the microfluidics approach used to produce the pH-responsive systems, co-loaded with GLP-1 and iDPP4. The inner fluid consisted of modified PLGA and PSi nanoparticles and the enteric polymer dissolved in ethyl acetate. The antidiabetic peptide GLP-1 was loaded into the modified nanoparticles, whereas the enzymatic iDPP4 was dissolved directly in the inner fluid. The outer continuous fluid was an aqueous solution of F127 (2 % w/v) which could efficiently stabilize the oil/water (O/W) interface.

3. Materials and Methods

3.1. Materials and cell lines

GLP-1 was purchased from United Peptides (USA). iDPP4 (NVP DPP 728 dihydrochloride) was purchased from Tocris Bioscience (UK) and CPP R9 was purchased from GenicBio (China). PLGA 50:50 was obtained from Purac Biomaterials, Purasorb® PDLG 5004A, The Netherlands. Polyvinyl alcohol (PVA), medium molecular weight CS, 2-(N-morpholino)-ethanesulfonic acid (MES), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and glutaraldehyde were purchased from Sigma-Aldrich (USA). HPMC-AS was

obtained by Shin-Etsu (Japan). Pluronic® F127 was purchased from BASF (Germany). Hank's balanced salt solution (HBBS), phosphate buffered saline (PBS), Alexa Fluor® 488 hydrazine, Versene and heat inactivated foetal bovine serum (FBS) were purchased from Life Technologies (USA). Dulbecco's Modified Eagle medium (DMEM), Roswell Park Memorial Institute medium (RPMI 1640), L-glutamine, non-essential amino acids, penicillin (100 IU/mL) and streptomycin (100 mg/mL), ethylenediamine tetraacetic acid (EDTA) and trypsin–EDTA were purchased from HyClone (USA). Human colon carcinoma (Caco-2), lymphocytic Raji B cells and human gastric adenocarcinoma (AGS) cell lines were obtained from American Type Culture Collection (ATCC, USA), and the human colorectal adenocarcinoma modified with methotrexate (HT29-MTX) cells was kindly provided by Dr. T. Lesuffleur (INSERM U178, Villejuif, France).

3.2. Preparation of PLGA nanoparticles

Polymeric PLGA nanoparticles were prepared through modified solvent emulsification-evaporation method, based on the water-in-oil-in-water (w/o/w) double emulsion technique (Zhang *et al*., 2006). PLGA 50:50 was used for the preparation of polymeric nanoparticles. 100 mg of polymer were dissolved in 1 mL of ethyl acetate. Then, 100 µL of GLP-1 solution in MilliQ-water (2.5 mg/mL) was added to this solution and homogenised for 30 sec with 70% amplitude using a Vibra-Cell™ ultrasonic processor (Sonics®, Sonics and Matrials, Inc., USA). The primary emulsion (w/o) formed was then added into 4 mL of the surfactant, a 2% aqueous solution of PVA, and homogenised again for 30 sec with the same amplitude. The second emulsion formed (w/o/w) was finally added to 7.5 mL of surfactant and was left under magnetic stirring at 300 rpm for 3 h for solvent evaporation.

CS solution was prepared by dissolving CS in 1% (v/v) acetic acid solution overnight under magnetic stirring. The pH was adjusted to 5.5, followed by filtration through a paper filter Millipore #2 and stored at 4ºC. To coat the nanoparticles with the CS, the formulation was added into a solution of CS after solvent evaporation, at a ratio of 2:1 (w/w) (CS:nanoparticles), and left overnight under magnetic stirring, allowing surface layer deposition of the polymer into the nanoparticles.

Nanoparticles were washed three times in MilliQ-water and separated through centrifugation, using an ultra-centrifuge (Optima™ TL, Beckman Coulter, USA), at 34,300 × *g* for 20 min. After centrifugation, the nanoparticles were re-dispersed in MilliQ-water and stored at 4°C for further analysis.

Drug-free nanoparticles were prepared according to this procedure but using MilliQ-water instead of GLP-1 solution as the internal aqueous solution.

3.3. Preparation of PSi nanoparticles

The free-standing PSi films were anodized using single crystal Si wafers <100> of p+-type with resistivity values of 0.01–0.02 Ωcm. The PSi was prepared by anodizing the wafers in hydrofluoric acid (38%)–ethanol mixture. The PSi nanoparticles production was conducted by applying three different current pulses to the Si-wafer. The hydrocarbonization treatment consisted of exposing the particles to a 1:1 (v/v) flow of N_2 and acetylene for 15 min at room temperature, followed by 15 min at 500 °C, and then cooling down to room temperature under N_2 flush. Wet ball milling was used to reduce the size of the hydrocarbonized free-standing PSi films, and the surface oxidation was minimized by using 1-decene as the milling liquid. The final size separation and exchange of the suspension media were done by centrifugation. The surface of the nanoparticles was further modified using undecylenic acid giving rise to undecylenic modified thermally hydrocarbonized PSi nanoparticles (Shahbazi *et al*., 2013, Kovaleinen *et al*., 2012).

For the GLP-1 loading, a ratio of 1:5 (w/w) was used for GLP-1-loaded PSi nanoparticles. The GLP-1 loading was performed by dispersing the PSi nanoparticles in a GLP-1 solution and stirring at room temperature for 90 min at 300 rpm. After that, the suspension was centrifuged at 27,600 × *g* for 5 min to remove the excess of free GLP-1. To coat the nanoparticles with CS through physical adsorption, the nanoparticles were added to a CS solution in a ratio of 2:1 (w/w) (CS:nanoparticles), and left overnight under magnetic stirring at 300 rpm. For the nanoparticles coated by physical adsorption with CS, the GLP-1 loading was performed prior to the coating in order to prevent the blockage of the nanoparticles pores by CS (Shrestha *et al*., 2014).

3.4. CPP conjugation to the CS-coated nanoparticles

The covalent conjugation between the free amine groups in the CS structure with the carboxylic group of CPP was made through the EDC/NHS coupling chemistry. For chemical conjugation, 1 mg of PLGA+CS nanoparticles and 300 µg of PSi+CS were separately dispersed in 1 mL of 10 mM MES containing EDC (final concentration 10 mM) and NHS (final concentration 17 mM) at pH 5.5. CPP was added to this dispersion in a ratio of 1:10 (CPP:nanoparticles) and the nanoparticles were allowed to conjugate overnight in the dark, stirring at 300 rpm originating PLGA+CS-CPP and PSi+CS-CPP, respectively. Afterwards,

the nanoparticles were collected by centrifugation at 34,300 × *g* for 20 min for PLGA+CS-CPP nanoparticles and at 27,600 × *g* for 5 min for PSi+CS-CPP, and then washed three times with MilliQ-water.

3.5. Fabrication of a Glass-Capillary Microfluidic Flow-Focusing Device

The microfluidics device consisted of an assembly of borosilicate glass capillaries on a glass slide, as previously described (Chu *et al*., 2007). One of the ends of the collection cylindrical capillary (World Precision Instruments, Inc.) was tapered using a micropipette puller (P-97, Sutter Instrument Co., USA) to a diameter of 20 µm. This diameter was further enlarged to *ca.* 80 µm using a microforge (P-97, Sutter Instrument Co., USA). The capillary had an inner diameter of approximately 580 µm and an outer diameter of approximately 1,000 µm and it was hydrophilic, preventing the wetting of the oil shell of the emulsion on its wall. Afterwards this cylindrical tapered capillary was inserted into the left end of the square capillary (Vitrocom, USA) and coaxially aligned. This square capillary had an inner dimension of 1000 µm. Two polyethylene tubes attached to syringes were added to the glass slide to allow the entering of the inner and out fluids at constant flow rates controlled by pumps (PHD 2,000, Harvard Apparatus, USA). A transparent epoxy resin (5 minute® Epoxi, Devcon) was used to seal the capillaries and the polyethylene tubes where required.

3.6. Enteric coating of nanoparticles using microfluidics

The GLP-1 loaded nanoparticles were encapsulated into a pH-sensitive polymer (HPMC-AS) using a double emulsion technique, through a microfluidic flow-focusing glass device (**Figure 11**) (Liu *et al.*, 2014). The device consisted of two types of glass capillaries with different diameters, in which the outer diameter of the inner cylindrical tapered capillary fitted the inner dimensions of the outer capillary, facilitating the alignment of their axes.

To prepare the co-drug loaded multifunctional systems, 0.5 mg of iDPP4 was added into 1 mL of 4% of HPMC-AS dissolved in ethyl acetate (oil phase). To this solution, 100 µL of MilliQ-water containing 20 mg of PLGA+CS-CPP or 200 µg of PSi+CS-CPP (water phase) were added dropwise and homogenised for 30 s using a Vibra-Cell™ ultrasonic processor (Sonics®, Sonics and Matrials, Inc., USA), originating to the first emulsion (w/o). This solution was then poured into a syringe to be injected in the microfluidic device as the inner fluid (oil phase). The outer fluid used was an aqueous solution of 2% Pluronic® F127 (water phase). The inner and outer fluids were both pumped into the microfluidic device in opposite

directions at 10 mL/h and 230 mL/h, respectively. This flow-focusing geometry forces the inner fluid to breakdown, forming the second monodisperse emulsion (w/o/w) droplets at the entrance orifice of the tapered cylindrical glass capillary. The droplets were collected in a cylindrical beaker containing 80 mL of an aqueous sucrose solution (100 mOsm/L) in order to facilitate the particles deposition. These particles were solidified through the diffusion of ethyl acetate to the external aqueous phase. As a result, multifunctional systems containing both GLP-1 loaded CS-CPP modified PLGA and PSi nanoparticles and iDPP4 into a pH-sensitive polymer were obtained, to form the H-PLGA and H-PSi respectively.

3.7. Particle characterization

The nanoparticles average size (*Z*-average), PdI and surface charge were analysed by dynamic light scattering using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd, UK).

The AE and LD of GLP-1 in the developed nanoparticles were calculated by the difference between the total amount of GLP-1 used to prepare the loaded nanoparticles and the amount of GLP-1 that remained in the aqueous phase, after the nanoparticles isolation by centrifugation, as described elsewhere (Araújo *et al*., 2014). The amount of GLP-1 was determined by high performance liquid chromatography (HPLC) (Agilent 1260, Agilent Technologies, USA), using a C_{18} column (4.6 × 150 mm, 5 µm, Supelco Discovery® C18, USA). The mobile phase consisted of 0.1% trifluoroacetic acid (pH 2.0) and acetonitrile initially set at the ratio of 70:30 (v/v), which linearly changed to 60:40 (v/v) as a gradient over 5 min. Afterwards, the ratio was kept constant for 5 more minutes. The flow rate was 1.0 mL/min and the injected volume of the sample was 75 µL. The column temperature was set at room temperature and the detection wavelength at 240 nm. The total area under the curve was used to quantify the GLP-1.

The morphology and surface topography, shape and size of the enteric encapsulated particles were assessed by SEM (SEM, Zeiss DSM 962, Germany).

3.8. pH-Sensitive response of the multifunctional particulate systems

After collection and washing of the particles, they were placed on the SEM supports with double sided carbon adhesive tape. The excess of water was removed with filter paper and different buffer solutions at pH values of 1.2, 4.0, 5.5, 6.0 and 6.8, were added on top of the particles for 2 h. For pH of 6.0 and 6.8, the 10 min time point was also analysed. After these time points, the excess of buffer solutions were removed with filter paper and the

particles were allowed to dry at room temperature overnight. Afterwards, the particles were sputter coated with platinum before visualizing under the SEM.

3.9. *In vitro* release studies

The multifunctional system particles, corresponding to 50 µg of GLP-1, were added separately to 14 mL of pH 1.2 buffer (50 mM KCl) to simulate the gastric fluid and to FaSSIF pH 6.8 (50 mM KH_2PO_4, 15 mM NaOH, 1.0% (w/v) pancreatin) to simulate the fasted state fluid of the intestinal milieu. At specific time points, aliquots of 750 µL were collected and the withdrawn volume was replaced with fresh medium, keeping the volume constant. All the collected aliquots were centrifuged at 27,600 × *g* and the supernatant was used for HPLC analysis in order to quantify the GLP-1 and iDPP4 released from the systems over time. All the tests were performed at 37 ºC and at 100 rpm under sink conditions.

GLP-1 was quantified by HPLC as described above. iDPP4 was quantified by the total AUC through HPLC (Agilent 1260, Agilent Technologies, USA), using a Kinetex 2.6u XB-C18 100A column (4.6 × 75 mm, Phenomenex®, USA). The mobile phase consisted of 0.025% of ammonium hydroxide (pH 9.5 adjusted with 50% of phosphoric acid) and acetonitrile set at the ratio of 82:18 (v/v) for 5.5 min. The flow rate was 1.0 mL/min and the injected volume of the sample was 5 µL. The column temperature was set at room temperature and the detection wavelength at 275 nm.

3.10. Cell culturing

AGS (passage numbers of 10–15) grew in a complete medium consisting of RPMI 1640 supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine, 1% (v/v) NEAA, and 1% (v/v) antibiotic–antimitotic mixture (final concentration of 100 U/mL Penicillin and 100 U/mL Streptomycin). Caco-2 (passage numbers of 31–40) and HT29-MTX (passage numbers of 20–50) cells grew separately in culture flasks in a complete medium consisting of DMEM supplemented in the same way as described before for RPMI. The cells were sub-cultured once a week using 0.25% trypsin–EDTA to detach the cells from the flasks and seeded at a density of 0.5×10^6 cells per 75 cm^2 flask. The culture medium was replaced every other day. Cells were maintained in an incubator (BB 16 gas incubator, Heraeus Instruments GmbH, Germany) at 37 ºC and 5- % CO_2 and 95 % relative humidity. Raji B cells (passage numbers of 26–35) were cultured in flasks with supplemented DMEM and with the same conditions as described above.

3.11. Cell viability studies

The viability tests were conducted using the AGS, Caco-2 and HT29-MTX cell lines. Typically, 100 µL of 0.5 × 10^6 cells/mL were seeded separately in 96-well plates and were allowed to attach overnight. Afterwards, the medium was aspirated and the wells were washed twice with 100 µL of pre-warmed HBSS–HEPES buffer (pH 7.4). After washing, 100 µL of the nanoparticle solutions at concentrations of 0.1, 0.25, 0.5 and 1 mg/mL were added to each well and the plates were incubated at 37 ºC for a period of 3 h for AGS, and for 12 h for Caco-2 and HT29-MTX cells. Afterwards, the plates were equilibrated at room temperature for about 30 min and then washed twice with 100 µL of fresh HBSS–HEPES buffer (pH 7.4) at room temperature. 50 µL of the reagent assay CellTiter-Glo® (Promega Corporation, USA) were added to 50 µL of HBSS–HEPES (pH 7.4) in each well. Negative (HBSS–HEPES buffer, pH 7.4) and positive (1% Triton X-100) control wells were also used and treated similarly as described above. The solutions were mixed and protected from the light for approximately 10 min on an orbital shaker at room temperature. Finally, the luminescence was measured using a Varioskan Flash plate reader (Thermo Fisher Scientific Inc., USA). All data sets were compared with a negative control, considered as 100% viability.

3.12. Cell–nanoparticle interactions

In order to visualize the nanoparticles, they were conjugated with Alexa Fluor 488™ (green fluorescence emission) after their production through the EDC/NHS coupling chemistry. The covalent conjugation was made between the free carboxylic groups of the nanoparticles and the free amine groups of Alexa Fluor 488™. For chemical conjugation, 1 mg of PLGA and 300 µg of PSi nanoparticles were separately dispersed in 1 mL of 10 mM MES containing EDC (final concentration 10 mM) and NHS (final concentration 17 mM) at pH 5.5 and activated for 2h in the dark. Afterwards, Alexa Fluor 488™ was added to this dispersion in a ratio of 1:70 (w/w) and the materials were allowed to conjugate for 2h in the dark, stirring at 300 rpm. After conjugation, the nanoparticles were collected by centrifugation at 34300 × g for 20 min for PLGA nanoparticles and at 27,600 × g for 5 min for PSi and washed with MilliQ-water. The other modifications were further made as described previously.

3.12.1. Confocal microscopy studies

The intestinal cell–nanoparticle interactions were evaluated through confocal microscopy using a Leica SP5 confocal microscope (Leica Microsystems, Germany). Caco-2 and HT29-MTX were co-cultured in a 90:10 ratio (Caco-2:HT29-MTX), in Lab-Tek 8-chamber slides (Thermo Fisher Scientific Inc., USA) and allowed to attach overnight (Araújo *et al*., 2014). Then, the cells were washed twice with pre-warmed fresh HBSS–HEPES buffer (pH 7.4). 50 µL of Alexa Fluor 488™ labeled nanoparticles at a concentration of 200 µg/mL were added to the cells and incubated at 37 °C for 3 h. After incubation, the cells were washed twice with pre-warmed fresh HBSS–HEPES buffer (pH 7.4). Afterwards, the plasma membrane was stained by adding 200 µL of CellMask™ Orange (Invitrogen, USA) and incubated for 3 min at 37 °C. The excess of staining solution was washed twice with pre-warmed fresh HBSS–HEPES buffer (pH 7.4) and the cells were fixed using 2.5% glutaraldehyde for 20 min.

3.12.2. Flow cytometry studies

The quantification of the nanoparticles that are associated to the cells was evaluated using flow cytometry. 1 mL of Alexa Fluor 488™ labeled nanoparticles at a concentration of 300 µg/mL were added to 0.7×10^6 Caco-2:HT29-MTX co-culture cells, in a ratio of 90:10, in pre-warmed HBSS–HEPES buffer (pH 7.4) and incubated at 37 °C for 3 h. Afterwards, cells were washed three times with HBSS–HEPES buffer (pH 7.4) and detached with Versene for the measurements. Then, the cells were re-suspended in basic sorting buffer that consisted of PBS (Ca/Mg^{2+} free) with 5 mM of EDTA, 25 mM of HEPES and 2% of FBS, to avoid cells aggregation, and analysed right after using a Beckman Coulter Galios™ Flow Cytometer with a laser excitation wavelength of 488 nm. The results were analysed using the software Kaluza Analysis Version 1.3.

3.12.3. Permeability experiments

For the permeability experiments a triple co-culture cell model was used. For that, 7×10^4 cells/cm^2 of Caco-2 and HT29-MTX cells in a ratio of 90:10 were seeded in 12-Transwell® cell culture inserts and were allowed to grow and differentiate for 14 days with medium replacement every other day. Then, 1.0×10^5 Raji B cell were added to the basolateral chamber for 7 days more in order to induce the phenotype change of Caco-2 cells into M cells and to obtain a triple co-culture model (Araújo and Sarmento, 2013). The permeability experiments across the cell monolayers were performed in the apical-to-basolateral direction

in HBSS–HEPES (pH 7.4) at 37 ºC using an orbital shaker (100 rpm). After removing the cell culture medium, the Transwells® were washed twice with pre-warmed fresh HBSS–HEPES (pH 7.4) buffer and equilibrated for 30 min. Then, 0.5 mL of nanoparticles corresponding to an amount of 6 µg/mL of GLP and 40 µg/mL of iDPP4 were pipetted into the apical side of the inserts. At different time points (15, 30, 60, 120 and 180 min), 100 µL of samples were taken from the basolateral side of the inserts and the same volume of pre-warmed fresh HBSS–HEPES (pH 7.4) buffer was added to replace the withdrawn volume. Sample concentrations were quantified for GLP-1 by EIA GLP-1 Kit (Sigma-Aldrich®, USA), according to manufacturer's instructions and for iDPP4 by HPLC, as previously described. The integrity of the cell monolayers was checked before and after the permeability experiments by measuring the transepithelial electric resistance (TEER) using Millicell®-Electrical Resistance System (Millipore, USA).

3.12.4. DPP4 enzymatic activity

The enzymatic activity of the DPP4 was measure according to the manufacturer's instructions using a DPP4 Activity Assay Kit (Sigma-Aldrich®, USA). After the permeability experiments, the cells were washed once with pre-warmed HBSS–HEPES buffer (pH 7.4). Then, the cells were lysed with 100 µL of ice-cold Assay Buffer. After lysis, 10 µL of each sample was diluted with Assay Buffer to have a final volume of 50 µL. To each test samples 10 µL of the Assay Buffer was added. To the blank samples, 10 µL aliquots of iDPP4 were added, mixed well and incubated for 10 min at 37 °C. Afterwards, 40 µL of the Master Reaction Mix was added to each test sample and properly mixed and protected from the light. The samples were then incubated for 5 min at 37 °C before starting the fluorescent measurements (λ_{ex} = 360 and λ_{em} = 460). The plate was kept at 37°C and protected from the light, and the measurements were taken every 5 min for 1 h using a Varioskan Flash plate reader (Thermo Fisher Scientific Inc., USA).

3.13. Statistical analysis

All the experiments were performed in triplicate and represented as mean ± standard deviation (SD). A Student *t*-test and one-way analysis of variance (ANOVA) with Uniparametric and Bonferroni post-test (GraphPadPrism, GraphPad software Inc., CA, USA) were used to analyse the data, respectively. The level of significance was set at probabilities of $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

4. Results and Discussion

4.1. Characterization of the multifunctional systems

After the production of the nanoparticles and the CS–CPP surface functionalization, the nanoparticles were characterized in respect to mean size, PdI, surface charge, AE and LD, as shown in **Table 5**. Both the unmodified PLGA and PSi nanoparticles presented a *z*-average size of *ca.* 200 nm with a PdI below 0.1, and negative surface charges. In order to increase the interaction of the nanoparticles with the negatively charged intestinal mucus layer and to improve the permeability of the antidiabetic peptide GLP-1 across the intestinal cells, the nanoparticles were coated firstly with CS to form PLGA+CS and PSi+CS nanoparticles (Araújo *et al*., 2014; Chronopoulou *et al*., 2013; Wang *et al*., 2013). The CS-modified nanoparticles showed an increase in their size and conversion of the zeta-potentials to positive values, demonstrating the successful CS coating of the nanoparticles (Araújo *et al*., 2014; Chronopoulou *et al*., 2013; Guo and Gemeinhart, 2008; Wang *et al*., 2013). In order to improve the transcellular transport, the CS-coated nanoparticles were further modified by covalent attachment of polyarginine R9 CPP to the amine groups of the CS. CPP, such as R9, are small peptides of few acidic amino acid residues with a high positive charge (Wang *et al*., 2014). Such CPP are known to have a positive role in the translocation of some drugs across the intestinal epithelium, increasing their oral bioavailability (Bu *et al*., 2014; Chen *et al*., 2012; Kamei *et al*., 2008; Liu *et al*., 2013c; Morishita *et al*., 2007). Overall, when modified with CS and CPP (to form PLGA+CS-CPP and PSi+CS-CPP particles), the nanoparticles presented higher particle sizes than the unmodified ones, without significant change in the zeta-potential values (**Table 5**). Together with the size and charge, the similarity in the AE and LD values, indicated the high homogeneity of the nanoparticles from batch-to-batch (Araújo *et al*., 2014).

The CS-CPP modified nanoparticles were further encapsulated within an enteric polymer, HPMC-AS, through the microfluidics technique, for their protection, as well as for the protection of the CS and CPP, at the low pH conditions of the gastric environment, originating the H-PLGA and H-PSi particles. The microfluidics technique has brought revolutionary impact in the pharmaceutical technology field by manipulating nanoliter volumes, in micro-scalefluidic channels, with high precision and reduced consumption of reagents (Liu *et al*., 2015; Valencia *et al*., 2012). In contrast with the conventional production methods, which yield a polydisperse population with drug loading levels less than ideal, with the microfluidics technique the e fficiency of encapsulation is near 100%, the particles formed are stable, homogeneous and can be size-controlled (Liu *et al*., 2014; Zhang *et al*., 2014). Moreover, it can be applicable for diverse constituent fluids, with various chemical

compositions and to different cargos, making it a promising technique for the production of several drug delivery systems (Herranz-Blanco *et al*., 2015). The flow-focusing geometry used in this work is a droplet-based method widely used to produce different types of fluid entities on a continuous basis by applying an extensional coflow (Herranz-Blanco *et al*., 2015). In this method, fluids are forced through a narrow orifice where high shear and capillary instability break bubbles off the tip, forming monodisperse emulsion droplets (Zhang *et al*., 2014).

As shown by the scanning electron microscopy (SEM) (**Figure 21)**, the produced particles had a regular and spherical shape with smooth surface and size around 59.44 ± 8.01 µm. The SEM images also showed a homogeneous size distribution, suggesting the production of monodisperse particles by the microfluidic technique (Liu *et al*., 2014). The loading degree of GLP-1 was of 0.030 ± 0.007% and 0.7300 ± 0.0001 % for the PLGA and PSi systems encapsulated into HPMC-AS, respectively, and for iDPP4 of 1.00 ± 0.01% and 1.260 ± 0.008%, respectively. The iDPP4 encapsulation efficiency was 20 ± 5%. Considering the amount (mass) of iDPP4 in the final formulation and that the IC_{50} of the iDPP4 is of 14 nM, the amount of inhibitor present in the current formulation should be more than enough to be active and to inhibit the enzyme activity efficiently. However, higher values can certainly be obtained in the final formulation, for example, by increasing the viscosity of the system (e.g., the viscosity of the HPMC-AS polymer) in order to retain strongly the iDPP4 in the polymer matrix, by increasing the osmolality of the collection solution or the solution viscosity, or by encapsulating the iDPP4 in the nanoparticles either together with the GLP-1 or alone.

Table 5. Characterization of the nanoparticles in respect to their size, PdI, surface charge (ζ-potential), AE and LD of GLP-1 of different nanoparticles. Results are presented as mean ± standard deviation, n ≥ 3.

Sample	Size (nm)	PdI	ζ-potential (mv)	AE (%)	LD (%)
PLGA	199.0 ± 4.1	0.08 ± 0.02	−23.6 ± 0.2	58.9 ± 6.9	0.14 ± 0.03
PLGA+CS	243.0 ± 2.2	0.26 ± 0.01	24.8 ± 2.7	59.7 ± 0.7	0.07 ± 0.01
PLGA+CS-CPP	277.2 ± 3.8	0.32 ± 0.02	21.6 ± 3.8	59.7 ± 0.7	0.07 ± 0.01
PSi	193.7 ± 3.0	0.09 ± 0.03	−16.3 ± 0.3	75.0 ± 0.6	15.00 ± 0.05
PSi+CS	282.9 ± 8.0	0.34 ± 0.02	19.2 ± 0.4	75.0 ± 0.5	7.50 ± 0.03
PSi+CS-CPP	320 ± 9.8	0.33 ± 0.02	19.1 ± 1.0	75.0 ± 0.5	7.50 ± 0.03

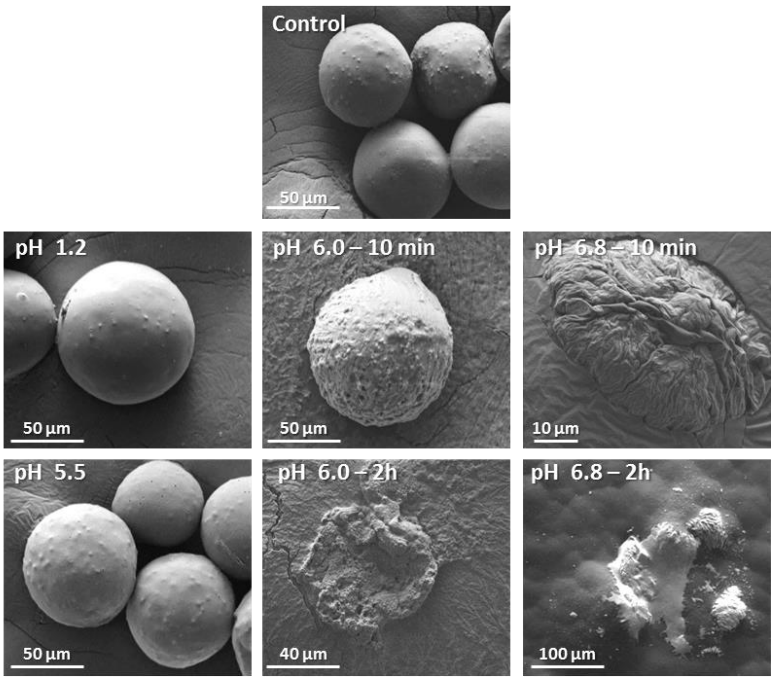

Figure 21. Dissolution behaviour of the CS-CPP modified nanoparticles encapsulated in the HPMC-AS polymer at different pH conditions of 1.2 and 5.5 for 2 h, and at pH 6.0 and 6.8 for 10 min and 2 h. The SEM images show the morphology of the prepared particles. The control group was composed by particles at pH 4.0. At pH values below 6.0 the structural integrity of the particles was maintained. At pH 6.0 and 6.8 the structure of the polymeric matrix was destroyed by the dissolution of the polymer.

4.2. pH-Sensitive response of the multifunctional systems

HPMC-AS is a polymer insoluble in acidic conditions and highly soluble at neutral or alkaline pH (pH > 6.0) (Liu *et al*., 2014). To evaluate whether the prepared nanoparticles were successfully encapsulated in the polymer and whether the multifunctional system could stand the harsh conditions of the stomach, the particles were observed under SEM at different pH conditions. As shown in **Figure 21**, at pH 1.2 and 5.5, the shape and surface morphology of particles were regular and smooth, similarly to the observed control particles, showing that the HPMC-AS encapsulation using the microfluidics technique was effective and that the nanoparticles were protected at the acidic conditions. When the multifunctional particulate system was in contact with buffer at pH 6.0, the polymer started to dissolve and the particles structure started to be compromised already after the first 10 min. After 2 h of incubation, the surface of the particles appeared rough with some visible holes as an indication for the dissolution of the polymer. At pH 6.8, this behaviour was even more

pronounced with greater changes in the particles' structure during the first 10 min. After 2 h the polymer was completely dissolved. These results are in accordance with the pH responsive characteristics of the polymer, as described elsewhere (Liu *et al*., 2014; Zhang *et al*., 2014).

4.3. Cell viability studies

The viability studies were performed in three different cell lines at two different time points and using different concentrations of the particles. AGS is a gastric epithelial cell line originated from human gastric adenocarcinoma (Afshari *et al*., 2014), and the cell viability was assessed after 3 h of incubation with the particulate systems. Caco-2 and HT29-MTX are intestinal cell lines originated from colon adenocarcinomas, representing the enterocytes and mucus producing goblet cells (Antunes *et al*., 2013; Araújo and Sarmento, 2013), respectively, and their viability was measured after 12 h of incubation with the particles. Testing these three cell lines in the two different time points covered the maximum transit time that the particles would be in the stomach and intestine when orally administered, making this a suitable assay to understand whether there is any concentration or component dependent toxicity (Araújo *et al*., 2014; Shrestha *et al*., 2014). Non-modified nanoparticles, CS-CPP-modified nanoparticles, and CS-CPP modified nanoparticles further encapsulated with HPMC-AS particles were tested.

With regards to the PLGA systems, it was observed in the three cell lines studied, that none of the non-modified nanoparticles, CS-CPP modified nanoparticles and CS-CPP modified nanoparticles encapsulated with HPMC-AS presented toxicity at the tested concentrations, as depicted in **Figure 22**. PLGA is commonly accepted for its low cytotoxic and good biocompatibility and biodegradability properties, and has already been approved by the FDA for clinical use (Liu *et al*., 2013c; Shi *et al*., 2014). On the other hand, positively charged particles are usually described as interacting more with the surface of the cell membranes than the negatively charged particles, which may lead to higher cellular cytotoxicity (Kumari and Yadav, 2011; Verma and Stellacci, 2010). However, in this study, despite the positive surface charge of the CS-CPP modified nanoparticles, no decrease in cell viability was observed in all the cell lines tested. This indicates that the amounts of CS and CPP used did not trigger cytotoxicity reaction, and thus, could be considered as safe for oral drug administration purposes. Previously, some studies have also demonstrated that certain CPPs, including oligoarginine, did not cause any harm to the intestinal epithelial cells (Kilk *et al*., 2009; Morishita *et al*., 2007).

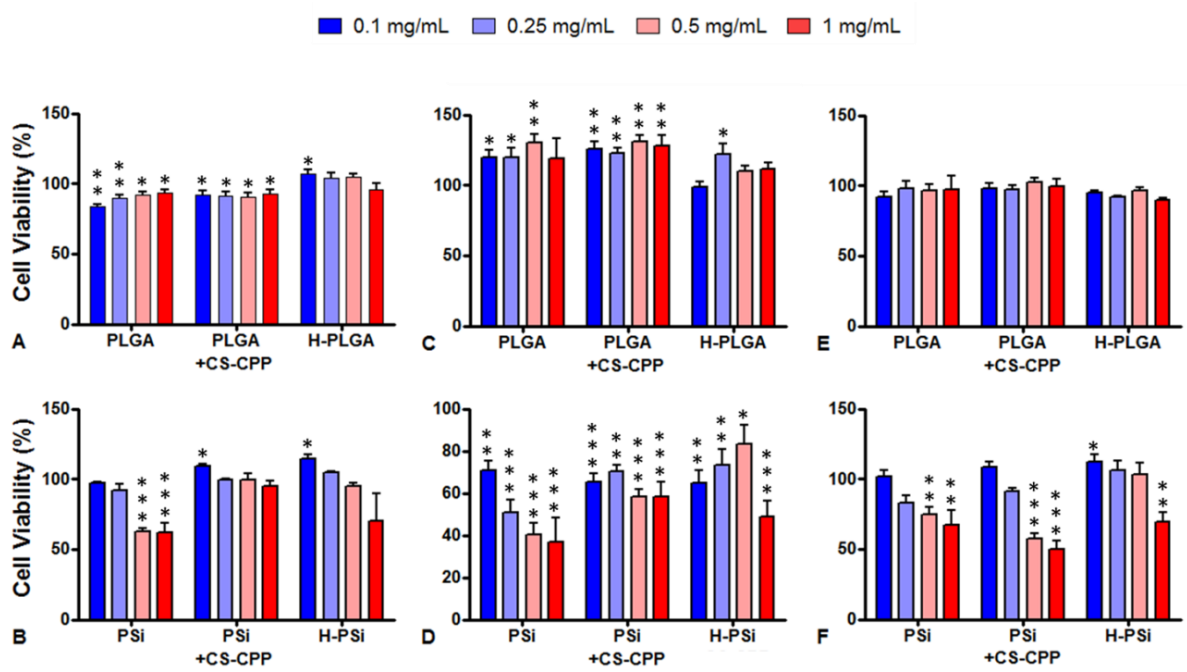

Figure 22. Cell viability levels of the gastric and intestinal cells after exposure to the particles assessed by the CellTiter-Glo® luminescence assay. (**A** and **B**) Viability of AGS after 3 h of incubation with different particles concentrations at 37 °C. (**C** and **D**) Viability of Caco-2 and (**E** and **F**) HT29-MTX cells after 12 h incubation with different particle concentrations at 37 °C. All the data sets were compared to the negative control (Hank's Balanced Salt Solution, HBSS) using a Student's *t*-test with an unpaired post-test. Error bars represent mean ± standard deviation ($n \geq 3$), and the difference probabilities were set at *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

HPMC-AS has been used as a pharmaceutical excipient and has a median lethal dose (LD_{50}) higher than 2.5 g/kg, which is much higher than the amounts used in our particle formulation (Hoshi *et al*., 1985). Moreover, the organic solvent used to dissolve the polymer, ethyl acetate (EA), is considered a rather non-toxic organic solvent (Liu *et al*., 2009), and it is believed that no residues of the solvent would be present in the final formulation. In fact, due to its high solubility in water, EA presented a fast diffusion rate to this solvent which allowed the fast solidification of the particles in the collecting solution, after their formation in the microfluidics device (Liu *et al*., 2009; Zhang *et al*., 2014).

For the PSi particles, it was observed that the non-modified nanoparticles, CS-CPP modified nanoparticles, and the CS-CPP modified nanoparticles encapsulated with HPMC-AS presented concentration dependent cytotoxicity values. The CS-CPP modified nanoparticles presented higher viability values than the non-modified ones with the exception

of the HT29-MTX cells. This behaviour may be related to the presence of CS on the surface of the nanoparticles, which would increase the interaction of the nanoparticles with the mucus secreting cells due to the mucoadhesive properties (Shrestha *et al*., 2014), as described elsewhere (Araújo *et al*., 2014). When the pH-sensitive polymer was used to encapsulate the CS-CPP modified nanoparticles, there were no statistically significant changes in the viability of the three cell lines. Although there was a tendency for improved cell viability values by the encapsulated nanoparticles compared to the non-encapsulated ones.

4.4. *In vitro* release studies

To confirm the successful protection of the CS-CPP modified nanoparticles and the loaded drugs from the acidic gastric conditions, as well as to predict the release profiles of the antidiabetic peptide GLP-1 and the enzymatic iDPP4, *in vitro* release studies were performed. In order to mimic the environment of the stomach, the study was conducted at pH 1.2 for 2 h. Similarly, to mimic the intestinal environment, the study was performed using simulate fasted state intestinal fluid (FaSSIF) at pH 6.8 for 6 h. The time points chosen are similar to the estimated time for the gastric and intestinal transit, respectively (Araújo *et al*., 2014).

The release profiles of GLP-1 from the H-PLGA+CS-CPP and H-PSi+CS-CPP particles at pH 1.2 and 6.8 are shown in the **Figures 23A** and **23B**, respectively. At pH 1.2, during the 2 h, the release of GLP-1 was less than 2% and 5% from the PLGA and PSi systems, respectively. However, at pH 6.8 the amount of GLP-1 released from particles was higher, reaching 40% release after 6 h for both the systems. For the H-PLGA+CS-CPP particles, the release was sustained along with the time, unlike in the case of H-PSi+CS-CPP release profile, in which there was a GLP-1 burst release, with 40% of the peptide released in the first 30 min and a very slow release thereafter. These biphasic release patterns dependent on the pH-value confirmed the controlled properties of encapsulation with the enteric polymer, and thus confirmed the fact that the CS-CPP modified nanoparticles and GLP-1 were indeed protected from the acidic conditions. These results are also in agreement with previous studies using the enteric polymer for oral drug delivery (Liu *et al*., 2014). The differences observed in the amounts that were released from H-PLGA+CS-CPP and H-PSi+CS-CPP over time were related with the characteristics of each nanoparticle and the methods used to load the GLP-1 in both of them. In PLGA nanoparticles, the GLP-1 was encapsulated inside of a polymer matrix, while in the PSi nanoparticles the GLP-1 was loaded mainly through physical adsorption in the pores of the nanoparticles which allowed a faster release (Araújo *et al*., 2014).

With regards to the enzymatic iDPP4 the release was not pH dependent, unlike to what was observed for GLP-1. As it can be observed in **Figures 23C** and **23D**, 80% of the iDPP4 was released at pH 1.2 during the first 30 min for both the formulations, reaching 100% release in less than 1 h. This can be explained by the very high solubility of the inhibitor in both aqueous solutions and in the organic solvents (Liu *et al*., 2009). The double emulsion technique could retain the aqueous solution in the organic solvent, and thus, retain the CS-CPP modified nanoparticles dispersed in water, until the complete polymer dissolution. However, this was not efficient in retaining the enzymatic iDPP4 and it started to diffuse into the organic solvent. When the particles were collected, the organic solvent started to diffuse to the water due to its high solubility. With the fast solidification process of the microdroplets, the iDPP4 was trapped in the outer layer of HPMC-AS matrix leading to the release of the enzymatic iDPP4 (Liu *et al*., 2009). Nevertheless, the enzymatic iDPP4 has been reported to be successfully administrated orally by adding it into the food and water, without compromising its activity, meaning that the enzymatic iDPP4 can resist the harsh conditions of the stomach (Mitani *et al*., 2002; Reimer *et al*., 2002).

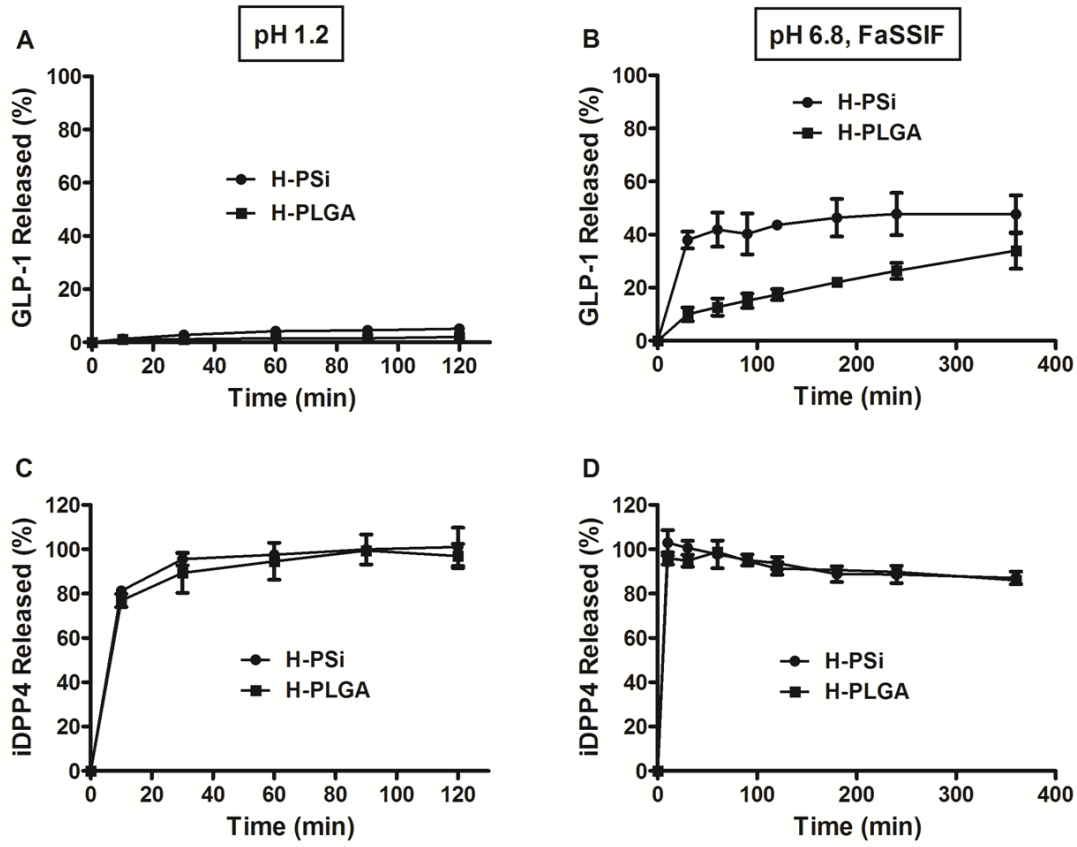

Figure 23. Release profiles of the GLP-1 and the enzymatic iDPP4 from H-PLGA and H-PSi particles (**A** and **C**) at pH 1.2 for 2 h and (**B** and **D**) at pH 6.8 for 6 h. All the experiments were performed at 37 °C and 100 rpm. The statistical analysis was done using a one-way analysis of variance (ANOVA) with a Bonferroni post-test. Error bars represent the mean ± standard deviation (*n* = 3).

4.5. Cell–nanoparticle interaction studies

Once in the small intestine, the CS-CPP modified nanoparticles were completely unconfined due to the dissolution of the pH-sensitive polymer. The interactions of the CS-CPP modified nanoparticles were analysed qualitatively using confocal fluorescence microscopy and quantitatively by flow cytometry experiments. For the confocal microscopy and flow cytometry experiments, the nanoparticles were labelled with Alexa Fluor® 488 hydrazine (green fluorescence emission) and the cell membranes were labelled with CellMask™ Red (red fluorescence emission). In line with the viability experiments, the Caco-2 and HT29-MTX cells, which are representative of the majority of the intestinal cells, were used to perform the cell–particle interaction studies. A ratio of 90:10 for Caco-2 and HT29-MTX cells, respectively, was used in order to mimic the physiological conditions of the

intestine (Araújo and Sarmento, 2013). The confocal fluorescence microscopy are shown in **Figure 24A**.

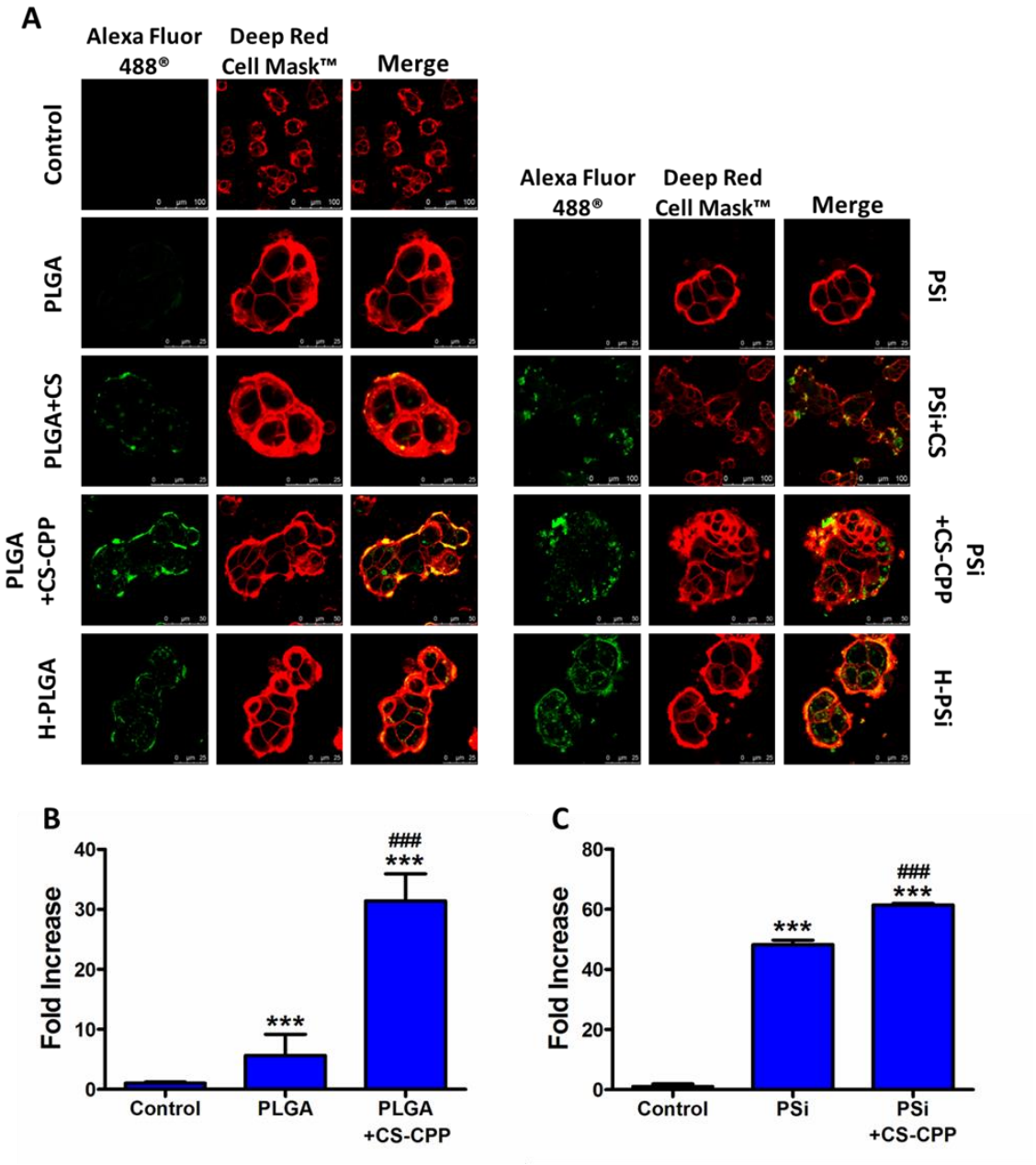

Figure 24. Interactions between the different nanoparticles and the Caco-2:HT29-MTX (90:10) co-culture after incubation for 3 h at 37 °C. (**A**) Confocal microscopy images of the cell membranes stained in red by CellMask® Red and the nanoparticles in green conjugated with Alexa Fluor® 488. Flow cytometry quantification of the interactions by (**B**) PLGA and (**C**) PSi nanosystems. The results were related to the fold increase of the percentage of cells interacting with the nanoparticles. Data sets were compared to the control (*p < 0.05, **p < 0.01, and ***p < 0.001) and between them ($^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, and $^{\#\#\#}p$ < 0.001) using a

Student's *t*-test with a uniparametric post-test. Error bars represent mean ± standard deviation ($n \geq 3$).

It was observed that for the unmodified nanoparticles there were no significant interactions with the cells for either the PLGA or the PSi nanoparticles. When the nanoparticles were modified with CS, the cell–nanoparticle interactions were greatly increased and many of the nanoparticles were observed in the close vicinity of the cell membranes. This increase in interaction could be due to the cationic nature and the mucoadhesive properties of the CS, causing strong electrostatic interactions with the mucus producing cells of the co-culture (Chen *et al*., 2013; Wang *et al*., 2013), as described elsewhere (Araújo *et al*., 2014; Shrestha *et al*., 2014). After the modifications with CS and CPP, the cell–nanoparticle interactions were even higher as compared to the non-modified nanoparticles, as more nanoparticles were associated to the cells' surface. In this case, the nanoparticles were interacting with the cell membranes and were also taken up by the cells. Even after the encapsulation with the enteric polymer, and once the polymer was dissolved at a higher pH, the nanoparticles presented a high capacity to interact and to be internalized by the cells.

Quantitative studies of the cell-particles interaction with flow cytometry demonstrated that the CS-CPP modified PLGA and CS-CPP modified PSi nanoparticles presented a 5.6-fold and 1.3-fold increase in the interaction with the intestinal cells in the co-culture, compared to the unmodified nanoparticles, respectively (**Figure 24B** and **24C**). The cationic nature of the CS and the CPP were important features that led to high cell–nanoparticle interactions (Shi *et al*., 2014).

4.6. GLP-1 permeability across the intestinal cell monolayers in the presence of iDPP4

The permeability of the antidiabetic peptide GLP-1 was studied for H-PLGA+CS-CPP and H-PSi+CS-CPP particles in the presence and absence of the iDPP4. As observed in **Figures 25A and 25B**, the GLP-1 permeation across the monolayers was higher for the H-PSi+CS-CPP particles than for the H-PLGA+CS-CPP particles. The difference between the amounts of GLP-1 permeated across the cell monolayers by the two systems may be due to the differences observed in the release profiles of GLP-1 loaded in both the particles. For the PSi system, it was observed a burst release of GLP-1 during the first 30 min, while for the PLGA system the release was more sustained over time. Thus, the amount of GLP-1 available to permeate across the cell monolayers in the presence of the PSi system was 2-

fold compared to the amount of the PLGA system, which justifies the observed permeability results after 3 h (Araújo *et al*., 2014). In the presence of the enzymatic iDPP4, the permeability of GLP-1 across the cell monolayers was even higher, with ≈ 1.5-fold increase for the PSi systems and ≈ 5-fold increase for the PLGA systems when compared to the permeability values without the enzymatic iDPP4 (**Figure 25C**). The synergistic effect of GLP-1 permeation in the presence the enzymatic DPP4 inhibitor is probably due to the inhibition of the DPP4 enzyme, which helped to protect the GLP-1 from degradation when permeating across the cell monolayer. Studies conducted in animal models have shown that when the iDPP4 was orally administrated, the plasma levels of intact and active GLP-1 were increased, which indicated that it was not rapidly degraded by the DPP4 enzyme (Reimer *et al*., 2002; Takasaki *et al*., 2004).

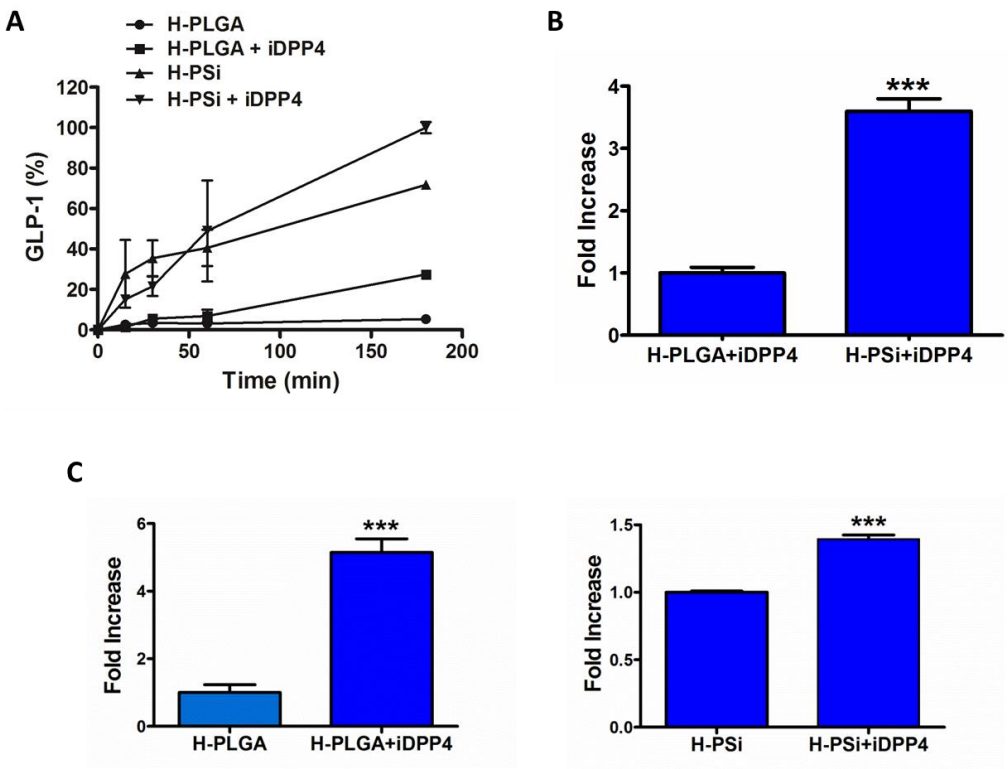

Figure 25. (**A**) Permeability profiles of GLP-1 from H-PLGA and H-PSi particles in the presence and absence of iDPP4. (**B**) GLP-1 permeability of H-PLGA and H-PSi across the Caco-2/HT29-MTX cell monolayers in the presence of iDPP4. (**C**) Differences between GLP-1 permeability across the Caco-2/HT29-MTX cell monolayers in the presence and absence of the iDPP4. The statistical analysis was done using a Student's *t*-test and one-way analysis of variance (ANOVA) with uniparametric and Bonferroni post-test. Error bars represent mean ± standard deviation ($n \geq 3$), set at probabilities of $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

When analysing the enzymatic activity of DPP4 (**Figure 26A**) after the 4 h incubation with the particles, it was observed that the enzymatic activity of DPP4 in the cells exposed to the inhibitor was significantly lower for both the PLGA (>20 µU/min) and PSi systems (40 µU/min) than in the cells with no exposure to the inhibitor (≈ 1062 µU/min). When compared to the free form of the inhibitor, the activity of the inhibitor loaded in the particles was not affected, and despite of no statistically significant difference, these values were lower than in the case of the free form of iDPP4. The similar permeability profiles of the enzymatic iDPP4 (**Figure 26B**) across the cell monolayers corroborate these results. It has been shown in *in vivo* studies that in the presence of iDPP4 the enzymatic activity was changed from 3.26 ± 0.19 mU/mL to 0.01 ± 0.03 mU/mL, and that the inhibition of the enzyme was dose dependent (Reimer *et al*., 2002; Takasaki *et al*., 2004).

Because iDPP4 has also been demonstrated has having a positive effect in decreasing the blood glucose levels, it will have an additional benefit of our system for future *in vivo* applications (Ahren *et al*., 2002; Reimer *et al*., 2002; Takasaki *et al*., 2004; Villhauer *et al*., 2002).

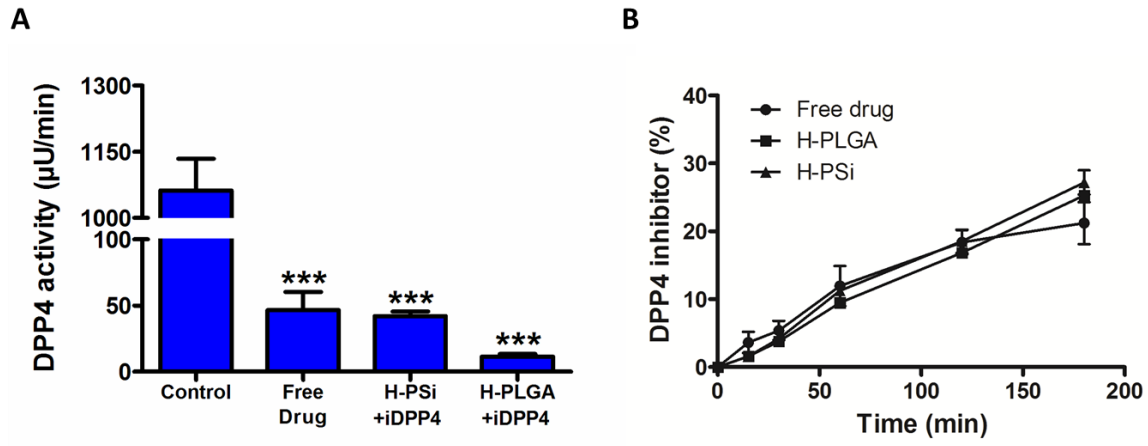

Figure 26. (A) DPP4 enzymatic activity after cellular incubation with particles loaded with the iDPP4. **(B)** Permeability profiles of DPP4 across the Caco-2/HT29-MTX cell monolayers in the free form and when loaded in both the multifunctional particulate systems. The statistical analysis was done using a Student's *t*-test and one-way analysis of variance (ANOVA) with uniparametric and Bonferroni post-test. Error bars represent mean ± standard deviation ($n \geq 3$), set at probabilities of $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

5. Conclusions

In this study, two novel multifunctional composite systems were prepared in a highly reproducible, efficient and reliable manner using the innovative microfluidics technique. The systems consisted of CS-CPP modified PLGA and PSi nanoparticles used as nanomatrices encapsulated into an enteric polymer, which were co-loaded with an antidiabetic peptide GLP-1 and an enzymatic iDPP4. The co-delivery of these two drugs was for the first time described. Due to the characteristics of the enteric polymer, the multifunctional composite systems were able to protect the CS-CPP modified nanoparticles and consequently prevent the premature release of the peptide and its degradation in the adverse conditions of the gastrointestinal tract. By precisely releasing the CS-CPP modified nanoparticles and subsequently the peptide in the upper intestine, the bioavailability of peptide and its permeation would be greatly improved. Moreover, due to the modifications of the nanoparticles, the interactions and the retention time of the nanoparticles with the intestinal cells were also greatly improved. It was also shown that the release of both the drugs had a synergistic effect since the presence of the enzymatic iDPP4 decreased drastically the activity of the enzyme, improving further the amount of active peptide permeated across the intestinal cells. Thus, taking particularly into account the GLP-1 peptide and the enzymatic iDPP4, these multifunctional particulate systems might be very promising for clinical applications in the therapy of T2DM, due to the potential to increase in GLP-1 half-life in an *in vivo* situation. Overall, due to the flexibility of the techniques employed here, and the multifunctional character of the particulate systems, they have potential to orally deliver sensitive biomolecules in combination with other drugs with different physicochemical properties.

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CHAPTER V

In vivo dual delivery of glucagon-like peptide-1and dipeptidyl peptidase 4 through PLGA-based composites prepared by microfluidics for diabetes therapy

This chapter was based in the following published paper:

- **Araújo, F.**, Shrestha, N., Gomes, M. J., Herranz-Blanco, B., Liu, D., Hirvonen, J. T., Granja, P. L., Santos, H. A. and Sarmento, B. 2016. *In vivo* dual-delivery of glucagon like peptide -1 (GLP-1) and dipeptidyl peptidase-4 (DPP4) inhibitor through composites prepared by microfluidics for diabetes therapy. Nanoscale. 8(20): 10706-13.

1. Abstract

Oral delivery of proteins is still a challenge in the pharmaceutical field. Nanoparticles are among the promising carrier systems for the oral delivery of proteins by increasing their oral bioavailability. However, most of the existent data regarding the nanosystems for oral protein delivery concerns *in vitro* studies, lacking *in vivo* experiments to evaluate the efficacy of these systems. Herein, a multifunctional composite system, tailored by droplet microfluidics, was used for dual delivery of GLP-1 and dipeptidyl peptidase-4 inhibitor (iDPP4) *in vivo*. Oral delivery of GLP-1 with nano- or micro-systems has been studied before, but the simulataneous nanodelivery of GLP-1 with iDPP4 is a novel strategy presented here. The T2DM rat model induced through the combined administration of streptozotocin and nicotinamide, a non-obese model of T2DM, was used. The combination of both drugs resulted in an increase in the hypoglycemic effects in a sustained, but prolonged manner, where the iDPP4 improved the GLP-1 therapeutic efficacy. Four hours after oral administration of the system, blood glucose levels were decreased by 44%, and were constant for another 4h, presenting half of the glucose area under the curve when compared to the control. An enhancement of the plasmatic insulin levels was also observed 6h after oral administration of the dual-drug composite system and, despite no statistic significant differences exists the amount of pancreatic insulin was also higher. These are promising results for the oral delivery of GLP-1 to be pursued further in a chronic diabetic model study.

KEYWORDS: oral delivery; glucagon like peptide-1; dipeptidyl peptidase-4; *in vivo*; type 2 diabetes *mellitus*; nanoparticles

2. Introduction

The development of therapeutic proteins has increased exponentially over the past decades (Leader *et al*., 2008). Owing to their natural origin, proteins and peptides have exquisite effectiveness, activity, specificity and relative lower toxicity, which has a significant impact in the treatment of numerous diseases (Fonte *et al*., 2015). However, due to their delicate structure, they must be administrated through parenteral routes. The use of proteins as therapeutic agents for oral delivery is still under achieved, which keeps the desired oral administration of proteins yet in its infancy (Li *et al*., 2013; Soudry-Kochavi *et al*., 2015).

With an expanding knowledge in nanomedicine the development of nanosized delivery systems is revolutionizing the pharmaceutical field, by considerably improving the therapeutic effect of most drugs and their bioavailability. Nanoparticles are also regarded as promising nanocarriers for oral protein delivery (Morishita and Peppas, 2006; Yu *et al*., 2015). These nanoparticles are formed by biomaterials that can be tailored towards the desired administration route and can further be associated with other molecules in order to improve their interaction with the target organ (Pereira *et al*., 2014). Nevertheless, despite the huge efforts in the development of nanoparticles, most of the existent data essentially addresses *in vitro* studies, lacking *in vivo* experiments to evaluate the efficacy of the developed nanosystems.

Herein, an innovative approach for T2DM therapy is proposed. Within a multifunctional tailorable composite system for dual-drug delivery previously described (Araújo *et al*., 2015), GLP-1 and a iDPP4 drug were combined in a single system, aiming to overcome the side effects associated to each one of them separately. Due to its incretin effect (insulin secretion after a meal, in a glucose-dependent manner) GLP-1 is one of the most promising therapeutic molecules for T2DM therapy, avoiding the well-known hypoglycemic effects of current drugs (Araújo *et al*., 2012; Baggio and Drucker, 2007). Nevertheless, GLP-1 has a very short half-life, being cleaved by the DPP4 enzyme in less than 2 minutes (Araújo *et al*., 2014). This system was assembled through the droplet microfluidics technique (Araújo *et al*., 2015) and it is based on the use of polymeric PLGA nanoparticles. These nanoparticles were claimed as adequate candidates to provide a protective, stable and biocompatible environment to the encapsulated peptides and proteins (Danhier *et al*., 2012; Fonte *et al*., 2015; Hosseininasab *et al*., 2014). The nanoparticles were functionalized with CS and a CPP and, as previously described by our group, showed high and strong interactions with intestinal cells *in vitro* (Araújo *et al*., 2015; Zhu *et al*., 2015). CS is a positively charged polymer used in large extent due to its advantageous characteristics of adhesion to negatively charged mucosae and cell membranes, thereby increasing cell permeability of intestinal cells by transiently opening tight junctions and affecting the

paracellular and intracellular pathways, without changing the junctional morphology or cause any damage to the cells (Canali *et al*., 2012; Chen *et al*., 2013). In turn, CPP has the ability to cross the cellular membranes without causing a cellular response, increasing the transcellular transport (Bechara and Sagan, 2013; Shi *et al*., 2014). We have previously shown *in vitro* that the dual-release of the peptide and the drug had a synergistic effect regarding GLP-1 permeability. In the presence of the iDPP4, the activity of the enzyme responsible for degrading GLP-1, thus considerably decreasing its bioavailability, was drastically reduced, further improving the amount of active peptide permeated *in vitro* across the intestinal cell monolayers (Araújo *et al*., 2015). Additionally, using the microfluidics system, the functionalized nanoparticles were encapsulated within an enteric polymer - hydroxypropylmethylcellulose acetylsuccinate (HPMC-AS) - conferring pH-sensitivity to the system and thus enabling the release of the nanoparticles only in the simulated intestinal conditions (Liu *et al*., 2014; Zhang *et al*., 2014). This synergetic effect was also described for other carriers (Yuan *et al*., 2015; Zhao *et al*., 2015). Both PLGA and HPMC-AS polymers are approved by FDA for parenteral and food administration, respectively. Previous studies also show that they are extensively used in the drug delivery field without toxicity effects after acute and chronic administrations (Araújo *et al*., 2015; Danhier *et al*., 2012; Hoshi *et al*., 1985; Shrestha *et al*., 2015).

Pursuing these promising results, in the present work the developed system was orally delivered to T2DM rat models, induced by the combination of STZ and nicotinamide drugs. Blood glucose, plasmatic insulin levels, and insulin pancreatic content were quantified over time during this study**.**

3. Materials and methods

3.1. Materials

GLP-1 acetate (7-37, MW 3355.7 Da) was purchased from United Peptides (USA). iDPP4 (NVP DPP 728 dihydrochloride, MW 375.77 Da) was purchased from Tocris Bioscience (UK) and CPP R9 was purchased from GenicBio (China). PLGA 50:50 was obtained from Corbion Purac, Purasorb® PDLG 5004A, The Netherlands. Polyvinyl alcohol (PVA), medium molecular weight CS, 2-(N-morpholino)-ethanesulfonic acid (MES), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), STZ, nicotinamide and isoflurane were purchased from Sigma-Aldrich (USA). HPMC-AS was obtained from Shin-Etsu (Japan). Pluronic® F127 was purchased from BASF (Germany). Lancet Unistik 2 Normal Fixed Depth Lancet Needle 2.4 mm 21 Gauge were purchased from

Owen Mumford, Ltd (France) and FreeStyle Precision Blood Glucose Test Strips from Abbott Diabetes Care (Portugal).

3.2. Preparation of GLP-1 loaded PLGA-CS nanoparticles

PLGA nanoparticles were produced based on the water-in-oil-in-water (w/o/w) double emulsion technique, through the modified solvent emulsification**.**evaporation method, using 2% of PVA as surfactant, as described elsewhere (Araújo *et al*., 2014; Araújo *et al*., 2015)**.** Afterwards, in order to functionalize the nanoparticles with CS, the formulation was added into a CS solution at a ratio of 1:2 (w/w), regarding the solid content of the solutions, and left overnight under magnetic stirring (Araújo *et al*., 2015)**.**

3.3. CPP conjugation to the CS-functionalized nanoparticles

EDC/NHS coupling chemistry was used to covalently conjugate the free amine groups in the CS structure with the carboxylic group of CPP, as previously described (Araújo *et al*., 2015). Briefly, the CS-functionalized nanoparticles were dispersed in MES solution containing EDC and NHS (pH 5.5.). CPP was then added to this dispersion in a ratio of 1:10 (CPP:nanoparticles, w/w) and the conjugation occurred overnight in the dark under 300 rpm stirring, forming PLGA+CS-CPP.

3.4. Characterization of nanoparticles

The nanoparticles were characterized in respect to their average size (Z-average), polydispersity (PdI) and surface charge zeta (ζ)-potential) by dynamic light scattering using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd, UK).

The AE and LD of GLP-1 were calculated by the difference between the total amount of GLP-1 used to prepare the nanoparticles and the amount of GLP-1 that remained in the aqueous phase after the nanoparticles isolation by centrifugation at 20,000 × *g* for 30 min at 4°C. The amount of GLP-1 was determined by high performance liquid chromatography (HPLC), as described elsewhere (Araújo *et al*., 2015).

3.5. Microfluidics enteric encapsulation of nanoparticles

The GLP-1 loaded nanoparticles were encapsulated within the HPMC-AS pH-sensitive polymer, loaded with the iDPP4, using a double emulsion technique through a microfluidic flow-focusing glass device. The preparation of the co-drug loaded multifunctional systems was previously described in detail (Araújo *et al*., 2015). The modified nanoparticles encapsulated in the HPMC-AS polymer are defined as H-PLGA particles.

The shape, size, morphology and surface topography of the enteric encapsulated particles were assessed by scanning electron microscopy (SEM, Zeiss DSM 962, Germany). The AE of the iDPP4 was calculated by dissolving the enteric encapsulated particles in a pH 7.4 solution. The amount of iDPP4 was determined by HPLC, as described elsewhere (Araújo *et al*., 2015).

3.6. Type 2 diabetic animals

Male Wistar rats, 7-weeks old, weighing 150-200 grams, obtained from Harlan Laboratories, Inc. (Spain) were used for the study. They were maintained in standard laboratory conditions (12h light/dark cycles, temperature of 21 ± 2°C and relative humidity of 35% to 60%). They were fed with standard pellet and water ad libitum.

The rats were randomly divided into 6 groups, with 5 animals per group. The groups were named according to the different formulations that were given as oral gavage: group 1 – normal animals (control group of normal rats and with no T2DM induction); group 2 – phthalate buffer solution at pH 4.0 (control group); group 3 – GLP-1 and iDPP4 aqueous solution; group 4 – H-PLGA empty particles; group 5 – H-PLGA particles loaded with GLP-1 (H-PLGA-GLP-1); and group 6 – H-PLGA particles loaded with GLP-1 and iDPP4 (H-PLGA-GLP-1-iDPP4) (study group). The amount of administered particles was equivalent to a GLP-1 content of 200 µg/kg of weight of the rat. This dose was chosen taking into account the administration doses of the GLP-1 analogs that are in the market [liraglutide (Victoza®) and exenatide (Byetta®)], which is around 20-30ug/kg and previous study performed by Huotari and co-workers where an amount of GLP-1 of 50 µg was s.c. administrated per mouse (Huotari *et al*., 2013).

Animal experiments were approved by the Local Ethics Committee at the University of Porto and conducted under the guidelines and recommendations of FELASA and the European Directive 2010/63/EU.

Induction of T2DM in animals, with exception of group 1, was made in overnight-fasted rats, by an intraperitoneal (i.p.) injection of 120 mg/kg of nicotinamide and, 15 min after, 60 mg/kg of STZ. STZ was freshly dissolved in citrate buffer (0.1 M; pH, 4.5) and

nicotinamide was dissolved in normal physiological saline buffer (pH 7.4), maintained on ice prior to use (Masiello *et al*., 1998).

Glucose tolerance was determined by the intraperitoneal glucose tolerance test (IPGTT) 3 days after the T2DM induction. Overnight-fasted animals were administered i.p. with a glucose solution (2 g/kg). Blood samples were taken by puncturing with the help of a lancet from the tail tip at different time points (-15, 30, 60, 90, and 120 min) after glucose administration. Blood glucose was measured using a glucometer Precision Xtra (Abbott Diabetes Care, Portugal) by placing a small drop of blood on a new test strip and recording the measurements.

3.7. Hypoglycemic effect *in vivo*

After proving the successful T2DM induction through the IPGTT experiments, different formulations were orally administrated in a phthalate buffer (pH 4.0), according to the groups defined in the previous section and blood glucose and insulin levels in plasma and pancreas were measured.

3.7.1. Blood glucose measurements.

The blood samples were withdrawn from the tail vein and blood glucose levels were measured for 8h at different time points (0, 0.5, 1, 2, 4, 6 and 8h) after administration. The AUC over 8h was calculated for each group. The total hypoglycemic decrease (HD%) in serum glucose levels were calculated as follows (Jin *et al*., 2009; Li *et al*., 2013).

3.7.2. Plasmatic insulin measurements.

At the time points of 0, 2 and 6h after administration, the withdrawn blood was collected into eppendorf tubes containing 0.5 M of EDTA (10% of the final volume) to prevent the blood clotting. The samples were centrifuged at 5,400 × *g* for 10 min at 4°C. The supernatants were collected and stored at –20°C until further studies. Insulin quantification was made according to the manufacturer's instructions using a rat Insulin Elisa kit from Mercodia (Sweden).

3.7.3. Pancreatic insulin content determination.

At the end of the study, the animals were sacrificed by cervical dislocation, after isoflurane anesthesia, and the pancreases were extracted. The pancreases were isolated by removing the fat and the connective tissues, and they were weighed. Then, they were placed separately in 3 mL of ice cold acid ethanol (0.18 M HCl in 70% ethanol) and kept on ice. Pancreases were further homogenized with a probe sonicator and 3 mL more of acid ethanol were added into the tube of the tissue homogenate and stored overnight at 4°C. Afterwards, the samples were centrifuged at 3500 rpm for 45 min at 4°C and the supernatant was transferred to another tube to be stored at –20°C. These steps were repeated twice more and the supernatants from the different centrifugations were collected together. Before the quantification of the insulin content, using the Rat Insulin Elisa kit from Mercodia (Sweden), samples were left at room temperature, mixed by vortexing, and diluted 1000–1500 times.

3.8. Statistical analysis

All the experiments were performed in triplicate and represented as mean ± standard deviation (SD). A Student t-test and one-way analysis of variance (ANOVA) with unpaired and Bonferroni post-test (GraphPadPrism, GraphPad software Inc., CA, USA) were used to analyze the data, respectively. The level of significance was set at probabilities of $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

4. Results and Discussion

4.1. Characterization of nanoparticles

As previously demonstrated by our group, CS is able to increase the permeability of the antidiabetic peptide GLP-1 across the intestinal cells (Araújo *et al*., 2014; Shrestha *et al*., 2014). Due to its positive surface charge, CS conferred a positive charge to the nanoparticles, which increases the interaction with the negatively charged intestinal cells and mucus layer. Additionally, CPP was also conjugated to the CS-functionalized PLGA nanoparticles, further enhancing the intestinal permeability of the GLP-1-loaded CS-modified nanoparticles (Araújo *et al*., 2015; Kamei *et al*., 2008; Liu *et al*., 2013; Zhu *et al*., 2015).

After production and CS-CPP surface functionalization, the PLGA nanoparticles were characterized on the basis of the different physicochemical parameters that are known to

have an impact in their interaction with cells and in the drug delivery (Verma and Stellacci, 2010). The mean size, PdI, surface charge (ζ-potential), AE and LD of GLP-1 were evaluated, as shown in **Table 6**. Comparing the non-modified nanoparticles with the CS-CPP conjugated nanoparticles, an increase in the size from 174 ± 5 to 351 ± 4 nm, an increase in PdI and an inverse of charge from –20 ± 2 to 40 ± 0 mV was observed, proving the efficient modification of the prepared PLGA nanoparticles (Araújo *et al*., 2014; Araújo *et al*., 2015). Due to the adding of CS-CPP to the system, the final mass was increased resulting in the decrease of the LD from 0.017 ± 0.03 to 0.08 ± 0.01. However, despite these surface modifications, the AE did not significantly change (approximately 70%).

Table 6. Characterization of the nanoparticles in respect to their size, PdI, ζ-potential, AE and LD of GLP-1. Results are presented as mean ± SD (n ≥ 3).

	Size (nm)	**PdI**	**ζ-potential (mV)**	**AE (%)**	**LD (%)**
PLGA	174.4 ± 4.9	0.120 ± 0.045	- 20.0 ± 1.5	69.5 ± 10.3	0.17 ± 0.03
PLGA+CS	286.7 ± 5.5	0.188 ± 0.015	34.7 ± 2.8	≈ 69.5 ± 10.3	0.08 ± 0.001
PLGA+CS-CPP	351.3 ± 3.5	0.210 ± 0.024	40.0 ± 0.1	≈ 69.5 ± 10.3	0.08 ± 0.001

Further encapsulation of the CS-CPP modified nanoparticles with a pH-sensitive HPMC-AS polymer was made using the droplet microfluidics technique (Liu *et al*., 2014; Zhang *et al*., 2014), to form H-PLGA particles. These particles presented sizes around 60 ± 7 µm with a regular and smooth surface and a spherical shape (**Figure 27**). The similarities between the particles are characteristic from the microfluidic production technique, which originates uniform structures, with the additional advantage of providing an AE of near 100% (Araújo *et al*., 2015; Liu *et al*., 2014; Zhang *et al*., 2014). The iDPP4 AE was 21 ± 4 %. Since HPMC-AS is only soluble at pH 6.0 or higher, at low pH conditions of the gastric milieu, the particles will remain intact protecting the integrity of the CS-CPP modified nanoparticles (Araújo *et al*., 2015; Liu *et al*., 2014).

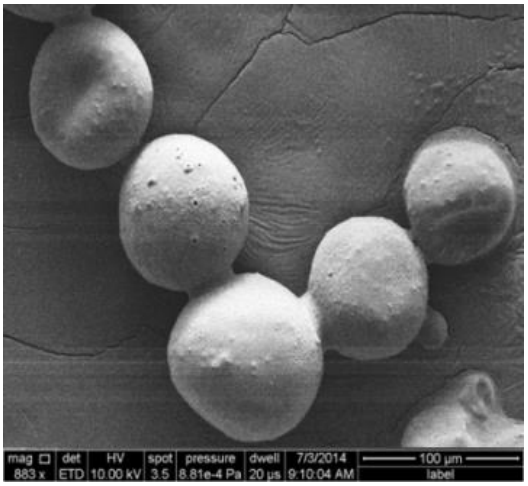

Figure. 27. SEM image of CS-CPP modified PLGA nanoparticles encapsulated in the HPMC-AS polymer (H-PLGA particles).

4.2. *In vivo* assessment of antidiabetic effect

A non-obese T2DM rat model induced by the administration of STZ combined with nicotinamide, was firstly proposed by Masiello and co-workers in 1998 (Masiello *et al*., 1998). This model was described as being the most suitable to study the biochemical and pharmacological antidiabetic drug effects, and was previously used to test GLP-1 analogs (Badole *et al*., 2013; Chen *et al*., 2013; Ghasemi *et al*., 2014). It is based on the protective effects of nicotinamide against the β-cytotoxic effects caused by the STZ, a widely used drug to induce diabetes *mellitus* in rodents (Masiello, 2006). This model presents a number of features similar with T2DM, as described in detail elsewhere (Ghasemi *et al*., 2014). Briefly, it provides a stable and moderate hyperglycemia, does not require exogenous insulin for animals to survive, results in glucose intolerance, reduction of β cells and presence (although impaired) of glucose-stimulated insulin secretion (Palsamy and Subramanian, 2008).

After 3 days of STZ–nicotinamide administration, a glucose tolerance test (IPGTT) was performed to verify the efficacy of the induction of the T2DM (Ghasemi *et al*., 2014). The glucose tolerance test was used to assess the ability of the body to metabolize glucose, and thus, to detect disorders in glucose metabolism. After the i.p. administration of glucose, the blood glucose levels were measured for 2h in the different groups. As shown in **Figure 28A**, the normal animals group had a rapid increase in the blood glucose levels 30 min after glucose administration, but the levels reached normal values 60 min after glucose administration and were constant, thereafter, *i.e*., the animals were tolerant to the glucose (Nayak *et al*., 2014). In contrast, the other groups could not recover from the glucose administration. There was a maximum peak between 30 to 60 min, similar to the normal

animals, but the blood glucose levels never reached the normal values in the 2h period after glucose injection. As shown in **Figure 28B**, all the groups presented a higher and statistically significant AUC compared to the normal animals group, in the period of -15–120 min of the study, which indicates that all the animals were intolerant to the glucose, and thus, were considered as presenting T2DM (Nayak *et al*., 2014).

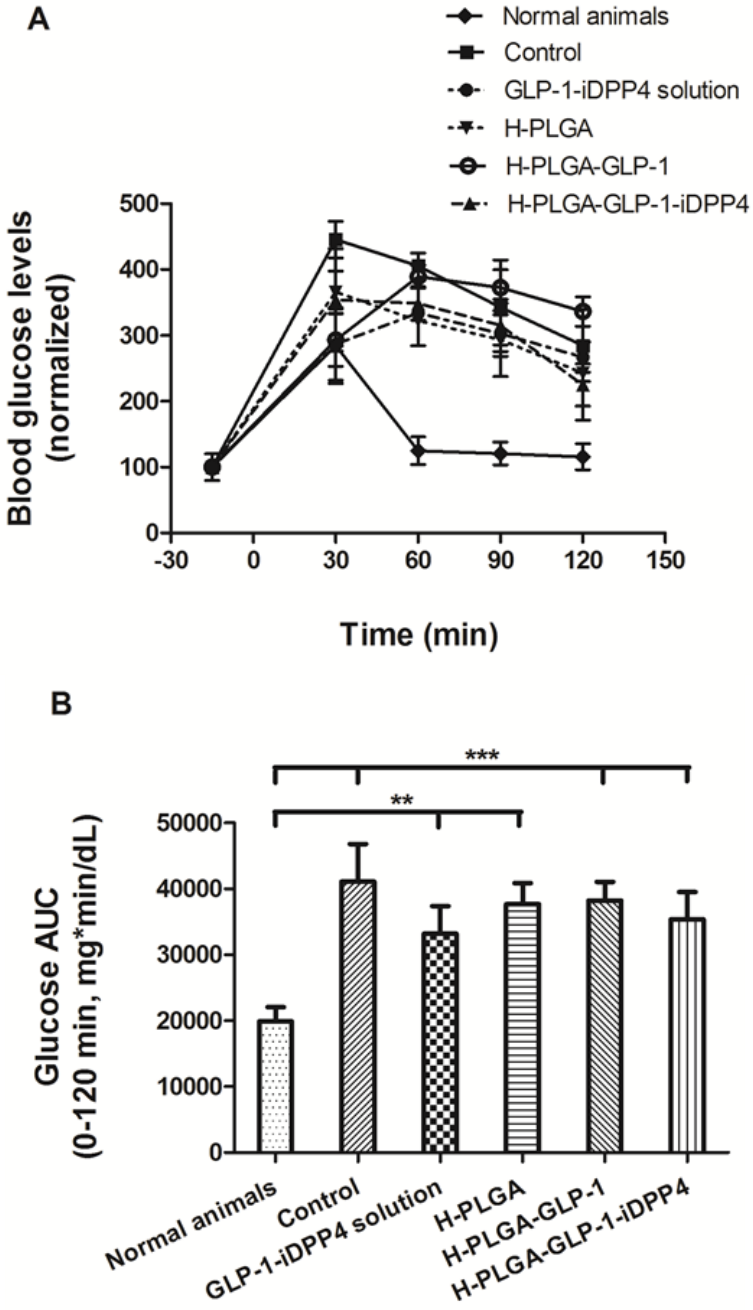

Figure 28. (**A**) Blood glucose levels of T2DM-induced rats following i.p. administration of a glucose solution (2 g/kg). The values were normalized by the normal animals group. Results are presented as mean ± SD (n = 5). (**B**) Blood glucose AUC in the period of -15–120 min after glucose administration. Results are presented as mean ± SD (n = 5). The levels of significance were set at probabilities of $^{**}p < 0.01$, and $^{***}p < 0.001$, as compared with normal animals group.

To evaluate the efficacy of our developed composite system, different formulations were orally administrated to the animals through gavage, as previously described in the Materials and Methods section. The drug-loaded H-PLGA particles (H-PLGA-GLP-1-iDPP4) showed remarkable decreased in the hyperglycemic effects (decrease in the blood glucose levels) in a sustained and prolonged manner. 2h after administration, the blood glucose levels decrease significantly, remaining low until the end of the experiment (8h). In comparison with other groups, at 4, 6 (*$p < 0.05$) and 8h (***$p < 0.001$) after administration, results with H-PLGA-GLP-1-iDDP4 particles were statistically different (**Figure 29A**). The determination of glucose AUC of the whole experiment (0–480 min), shown in **Figure 29B**, proved that the GLP-1 and iDPP4 co-loaded particles had a statistically significant effect in decreasing the overall glucose levels comparing to the control and the pure drugs, as well as in comparison with the empty H-PLGA and H-PLGA-GLP-1 particles (**$p < 0.01$). The hypoglycemic decrease (HD%) presented in **Table 7** also gives consistency to the obtained results, showing a HD% of approximately 0 for the GLP-1-iDPP solution and H-PLGA-GLP-1, 4.3 ± 6.4 % for the empty H-PLGA particles, which is a negligible hypoglycemic efficacy (Jin *et al*., 2009), and 44.3 ± 4.0 % for the DPP4-loaded H-PLGA particles. This hypoglycemic efficacy was similar (Jin *et al*., 2009) or even higher (Nguyen *et al*., 2011; Youn *et al*., 2008) than the results obtained in other studies where GLP-1 analogs were tested. Since the analogs are resistant to the DPP4 enzyme activity, we can assume that the dual delivery of GLP-1 and iDPP4 was effective, and that they had a synergetic effect regarding the blood glucose levels, as previously described for the *in vitro* experiments (Araújo *et al*., 2015; Shrestha *et al*., 2015). Moreover, the functionalization of the PLGA nanoparticles improved the permeability of cells to the GLP-1 and contributed to the overall increase in efficacy (Araújo *et al*., 2014; Araújo *et al*., 2015).

Being a powerful insulinotropic peptide, GLP-1 stimulates pancreatic insulin secretion and release in a glucose-dependent manner. Even in a T2DM scenario, the insulin response to GLP-1 remains intact (Lynn *et al*., 2001), which means that after administration of GLP-1 in therapeutic dosages, the insulin secretory function can be restored in T2DM patients (Garber, 2011). This glucose dependency assures the safety of GLP-1 over other agents in the market that increase insulin secretion via glucose-independent mechanisms (Nguyen *et al*., 2011; Youn *et al*., 2008).

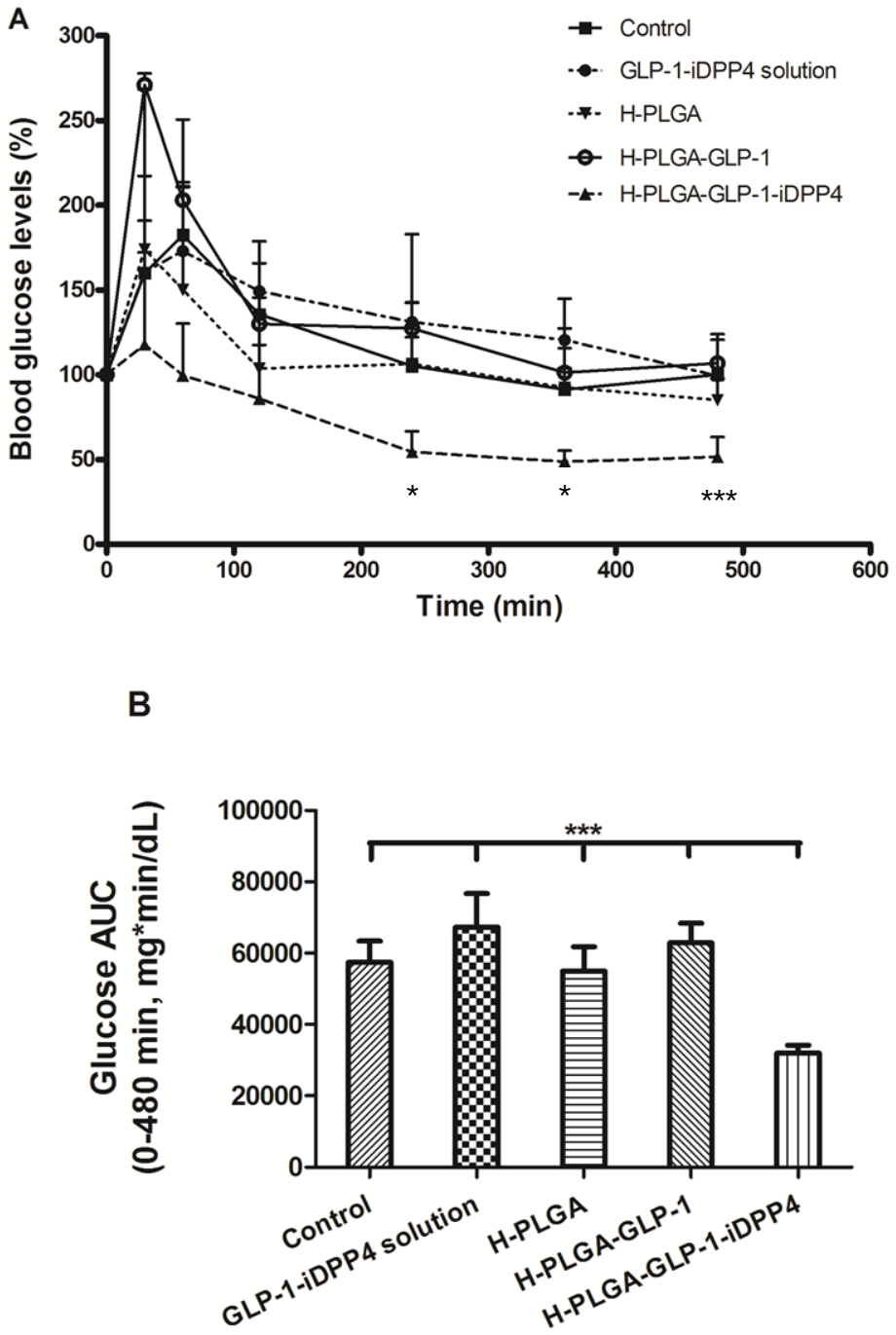

Figure 29. (**A**) Blood glucose levels of T2DM-induced rats following oral administration of phthalate buffer solution (control), GLP-1-iDPP4 solution, H-PLGA particles, H-PLGA-GLP-1 particles and H-PLGA-GLP-1-iDPP4 particles. Results are presented as mean ± SD (n = 5) (**B**) Blood glucose AUC in the period of 0–480 min after oral administration. Results are presented as mean ± SD (n = 5). The levels of significance were set at the probabiliy of ****p* < 0.001, as compared with the H-PLGA-GLP-1-iDPP4.

Table 7. Total hypoglycemic decrease (HD %) in serum glucose levels in the 8h experiment, regarding the control.

GLP-1-iDPP4 solution	H-PLGA	H-PLGA-GLP-1	H-PLGA-GLP-1-iDPP4
≈ 0	4.3 ± 3.6	≈ 0	44.3 ± 2.9

Thus, the plasmatic insulin levels were also measured for the time points of 0, 2 and 6h after oral administration. As can be depicted in **Figure 30A**, no differences between the groups were found for the initial times points (0 and 2h). However, at 6h after oral administration, the H-PLGA-GLP-1-iDPP4 particles presented significantly higher plasmatic insulin levels compared to the control, the oral GLP-1-iDPP4 solution, the H-PLGA empty particles and the H-PLGA-GLP-1 groups (***$p < 0.001$). These results are in accordance with the blood glucose level measurements where the blood glucose levels were lower at the time point of 6h (*$p < 0.05$) in comparison with the 0 and 2h time points. This is also in agreement with other study that showed an insulin increase in a slow, but prolonged manner along 8h (Nguyen *et al*., 2011).

The insulin pancreatic contents of the different groups after oral administration were also evaluated at the end of the 8h experiment, as shown in **Figure 30B**. The H-PLGA-GLP-1-iDPP4 group presented a higher amount of insulin compared with the other groups; however, this difference was only statistically significant regarding the control group. These results might be due to the single dose administration, while an increase in the insulin pancreatic content was reported to exist only in long-term studies (Moritoh *et al*., 2008, 2009; Reimer *et al*., 2002; van Genugten *et al*., 2012; Wu *et al*., 2012). Thus, despite promising, these results must be followed up in a chronic diabetic model system.

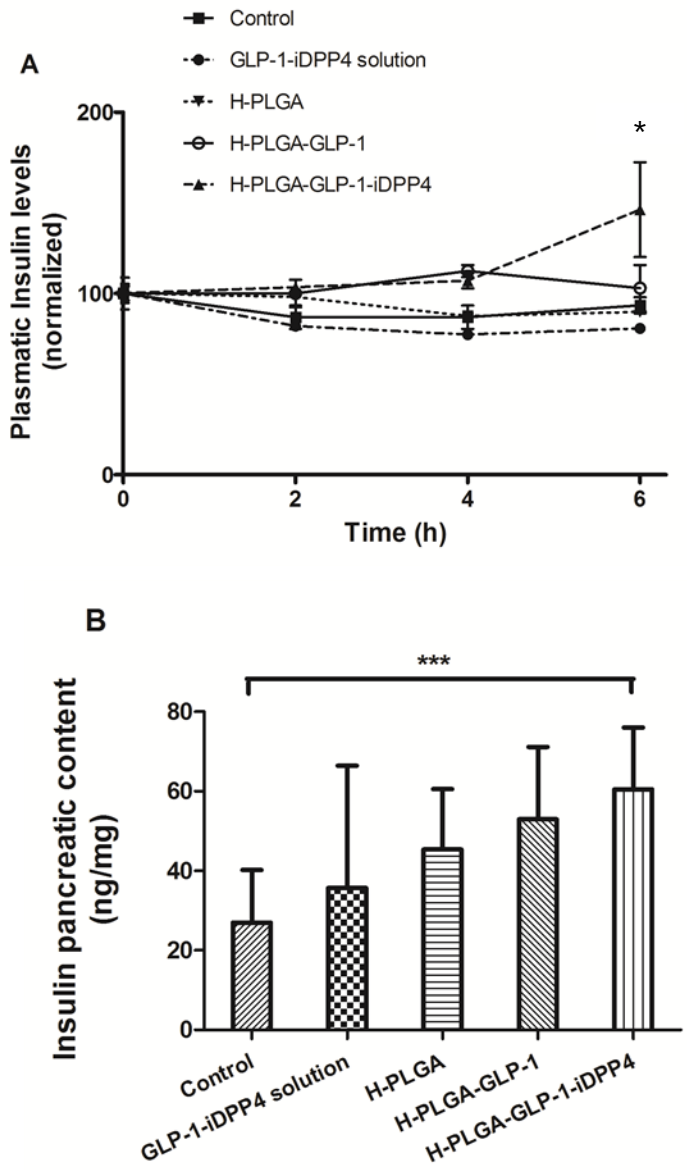

Figure 30. (**A**) Plasmatic insulin levels in T2DM-induced rats following oral administration of buffer solution (control), GLP-1-iDPP4 solution, H-PLGA empty particles and H-PLGA-GLP-1-iDPP4 particles. Results are presented as mean ± SD (n = 5). (**B**) Pancreatic insulin content 8h after oral administration of buffer solution (control), GLP-1-iDPP4 solution, H-PLGA empty and H-PLGA-GLP-1-iDPP4 particles. Data shown as mean ± SD (n = 5). The level of significance was set at the probability of ***$p < 0.001$ between the control group and the H-PLGA-GLP-1-iDPP4.

Overall, the combined administration of GLP-1 and iDPP4 resulted in an increase in the hypoglycemic effects after oral administration, namely a decrease of blood glucose levels and enhancement of the insulin secretion. A clear improvement of the therapeutic efficacy of GLP-1 was observed by the presence of the iDPP4 (Balkan *et al*., 1999; Green *et al*., 2005; Tian *et al*., 2010).

5. Conclusions

In this work, a dual-drug delivery multifunctional composite system was prepared through the highly reproducible microfluidics technique. The system was loaded with GLP-1 and iDPP-4 and tested *in vivo* in a non-obese T2DM rat model, induced by streptozotocin and nicotinamide. The combination of both GLP-1 and iDPP-4 resulted in an increase in the hypoglycemic effects in a sustained and prolonged manner. The blood glucose AUC was significantly lower than the control group, with a hypoglycemic decrease of 44%. An enhancement of the plasmatic insulin levels was also observed 6h after the oral administration of the system. These are very promising results towards the development of oral protein/peptide delivery systems for T2DM therapy.

6. Aknowledgements

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CHAPTER VI

Concluding remarks and future perspectives

1. Concluding Remarks

Materials science has been playing a paramount role in innovation and successful development of the biomedical field. Therapeutical approaches may beneficit from the modulation of payload delivery, provided by cutting-edge materials, in chronic and widespread diseases as type 2 diabetes *mellitus* (T2DM). As discussed in this thesis work, multistage platforms, considered as advanced delivery systems for the administration of peptides and proteins through the oral route, present various beneficial features. The specific engineering of carriers, namely by combining materials and tailoring their properties through functionalization, opened new avenues that potentiate mechanical, chemical and biological properties, and render positive characteristics to the materials. Additionally, modifying the surface of the particulate systems may contribute to the successful oral delivery of peptides and proteins in such a way that the traditional barriers along the gastrointestinal tract can now be considered as exciting opportunities for enhancing drug bioavailability.

In this work, particulate-based systems were developed as oral carrier systems for glucagon-like peptide-1 (GLP-1), aiming to enhance GLP-1 oral bioavailability. Among different materials (poly(lactic-co-glycolic acid) (PLGA), Witepsol E85 and porous silicon (PSi)), the PLGA and PSi nanoparticles modified with chitosan, with sizes around 200 nm and 360 nm, respectively, and positive charge, exhibited the best combinations of properties regarding the association efficiency (AE) of GLP-1, namely 60% for PLGA and 85% for PSi nanoparticles, and enhanced the cellular compatibility and interactions with intestinal cell lines. These nanoparticles were further functionalized with a cell penetrating peptide (CPP), and were encapsulated into hydroxypropylmethylcellulose acetylsuccinate (HPMC-AS) microparticles of *ca.* 60 µm, in which a dipeptidyl peptidase 4 (DPP4) (enzyme responsible to cleave, and hence, inactivate GLP-1) inhibitor, to enhance GLP-1 half-life, was co-loaded (AE of ca. 20%). The loading degree of GLP-1 was of 0.030 ± 0.007% and 0.730 ± 0.001 % for the PLGA and PSi systems encapsulated into HPMC-AS, respectively, and for iDPP4 of 1.00 ± 0.01% and 1.26 ± 0.01%, respectively. The smoth surface multistage composites were prepared in a highly reproducible, efficient and reliable manner using a microfluidics technique, taking into consideration a future scale up of the production. The use of the enteric polymer prevented the premature release of GLP-1 and its degradation at pH 1.2, which mimicks the gastric pH, and thus the adverse conditions of the gastrointestinal tract, since it only degrades at pH ≥ 6.0. Thus, a pH-responsive system efficient in protecting GLP-1 premature release was obtained. The functionalization of the nanoparticles with the CPP increased the permeability of GLP-1 and the interaction with the intestinal cells was even stronger, namely 5.6-fold for PLGA and 1.3-fold for PSi systems in comparison with non-

modified nanoparticles. It was also shown that the release of both GLP-1 and DPP4 inhibitor had a synergistic effect and that the DPP4 inhibitor further improved the amount of active peptide permeated across the intestinal cells by decreasing the activity of the DPP4 enzyme over 20-fold. The amount of GLP-1 permeated across a triple intestinal model increased 5- and ca. 1.5-fold for the PLGA- and PSi-based systems, respectively, in the presence of the DPP4 inhibitor, in comparison with systems without the DPP4 inhibitor. Moreover, the oral delivery of GLP-1 using the PLGA-based multisystem composites was assessed in an *in vivo* scenario, in a non-obese T2DM rat model. The composites combining GLP-1 and DPP4 inhibitor promoted a decrease in the hyperglycemic effects (blood glucose levels decreased 45%) in a sustained and prolonged manner (from 4 until, at least, 8h after oral administration). The blood glucose area under the curve was also significantly lower than the control group. An enhancement of the plasmatic insulin levels was likewise observed 6h after the oral administration of the system, as well as the pancreatic insulin content.

Overall, the multifunctional and multistage GLP-1 delivery system developed, based on the study of distinct biomaterials that were surface functionalized using mucoadhesion and permeation enhancers, and further encapsulated into a pH-sensitive polymer, revealed to increase the GLP-1 oral bioavailabilty after their oral administration, which are promising characteristics for clinical applications in the treatment of T2DM.

2. Future Perspectives

Multistage platforms for oral delivery of peptides and proteins are the product of cumulative research in the technology and pharmaceutical fields applied to modern medicine. Advancing the understanding of the design of these platforms is paving the way for significant progress to be achieved with respect to their synthesis, functionalization and future application in a myriad of worldwide diseases.

In this particular work, the development of new and "smart" delivery systems to orally deliver peptides was pursued, especially designed to overcome the barriers along the gastrointestinal tract. The promising results obtained opened new and exciting possibilities for exploration!

An interesting alternative strategy relies in the encapsulation of GLP-1 analogs instead of GLP-1. Those analogs, such as Exenatide (Byetta®) and Liraglutide (Victoza®), are claimed to have a longer half-life than GLP-1, thereby requiring lower amounts of administered formulation or higher efficiency for the same amount used here. However, these analogs are also known to cause some side-effects and thus strategies need to be envisaged to avoid them. Similarly to what was reported in this thesis, an alternative multistage delivery system could be developed, loaded with a drug molecule able to prevent those side-effects, in addition or replacing the DPP4 inhibitor.

To confirm the *in vivo* efficacy of the system developed and enhance its significance, more clinically relevant animal models need to be used, with increased similarity with the human, such as pig. Also, despite constituting a good model of T2DM, the STZ-nicotinamide induced rat model is only temporary since the animals' hyperglycemia starts dropping a few weeks after. Assessing the behavior of the developed multisystem composite in a chronic disease model, namely to understand its efficacy in controlling blood glucose levels during longer periods of time, would be of great relevance. The safety of the developed system should also be assessed in a chronic disease model study, including a detailed analysis of the immune response along with a thorough whole body histological evaluation to assess the systemic effects of regularly administered particles.

Another interesting aspect requiring optimization is the production of the systems. In the present work, particles were produced through a low yield and time-consuming microfluidics technique that cannot be scaled-up at an industrial level. However, the optimization of the microfluidics system may result in a continuous flux of microparticle production, as already explored by biomedical companies such as Dolomite Microfluidics and MiniFAB. Alternatives to explore include using different techniques to deliver the nanoparticles into enteric materials, or lyophilization of the nanoparticles using an optimized

protocol ensuring the bioactivity of GLP-1, followed by encapsulation into macroscopic capsules or pills, constituted by enteric coating materials. From an industrial point of view, strategies to reduce the production time and making possible the scaling-up of GLP-1 loaded nanoparticles would be of great relevance.

In a more general perspective, the flexibility of the techniques employed for the development of the system, coupled with the multifunctional character of the particulate system developed, indicate great potential to orally deliver other sensitive biomolecules such as other peptides or proteins and antibodies in combination with other drugs, with different physicochemical properties, such as the chemical drugs conventionally used in the clinical practice. The strategies and multistage delivery systems developed herein could be also of interest to orally deliver other molecules for different purposes than T2DM therapy, for instance in cancer therapy. Most of the drugs used in cancer therapy are expelled from cells by the P-glycoprotein (P-gp); using this multysistem, the drug can be co-loaded with a P-gp inhibitor increasing thus its absortion. Another potential application may be the co-administration of lumen enzyme or bacteria inhibitors that prematurely degrade active molecules prior to their absorption.

Overall, it is expected that drug delivery multistage platforms, as developed in the present thesis, could be validated and effectively applied to overcome physiological, technological and social limitations in biomedical fields.

APPENDIX

In vivo dual delivery of glucagon-like peptide-1 and dipeptidyl peptidase 4 through PSi-based composites prepared by microfluidics for diabetes therapy

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Similar as with the PLGA composite systems, also the PSi composite systems were tested *in vivo,* pursuing the promising *in vitro* results obtained and described in *Chapter IV.*

As previously shown, the PSi composites have the ability to sustain the release of GLP-1 in pH conditions below 6.0 due to the HPMC-AS polymer properties, which only dissolves at pH ≥ 6.0, and the dual release of GLP-1 and iDPP4 showed to have a synergetic effect in the permeability of GLP-1 through intestinal monolayers (Araújo *et al*., 2015).

The animal model used was a non-obese STZ and nicotinamide induced T2DM rat model (Araújo *et al*, 2016). Glucose tolerance was determined by the intraperitoneal glucose tolerance test (IPGTT) 3 days after the T2DM induction to verify its efficacy. Overnight-fasted animals were administered i.p. with a glucose solution (2 g/kg). Blood samples were taken by puncturing with the help of a lancet from the tail tip at different time points (−15, 30, 60, 90, and 120 min) after glucose administration. Blood glucose was measured using a glucometer Precision Xtra (Abbott Diabetes Care, Portugal) by placing a small drop of blood on a new test strip and recording the measurements. Results are shown in **Figure A1**.

The IPGTT results (**Figure A1A**) showed that the group of the normal animals had a rapid recover, with constant values subsequently, after an increase in the blood glucose levels, 30 min after glucose administration. This means that the animals were tolerant to glucose. In contrast, the other groups could not recover from the glucose administration and the blood glucose levels never reached the normal values in the 2 h period after glucose injection. These results were confirmed by the statistically significant increase in the AUC of all groups in comparison with normal animals, in the period of −15–120 min of the study, after oral administration, which indicates that all the animals were intolerant to glucose (**Figure A1B**). Hence the animals were considered as having the T2DM disease.

Next, and to evaluate the efficacy of the PSi developed composite systems, different formulations were orally administrated to different animal groups through gavage: i) buffer solution, ii) GLP-1 and iDPP4 in solution, iii) empty H-PSi and iv) H-PSi-GLP-1-iDPP4. Blood samples were withdrawn from the tail vein and the blood glucose levels were measured for 8h at different time points (0, 0.5, 1, 2, 4, 6 and 8h) after administration. The AUC over 8h was calculated for each group.

As seen in **Figure A2A**, the blood glucose levels of all the groups were very similar for all the measured time points, with no decrease in the values when compared to the initial ones. Moreover, comparing the AUC of all the groups (**Figure A2B**), none of them was statistically significant different from the other groups, corroborating the blood glucose levels results.

Being a powerful insulinotropic peptide, GLP-1 stimulates pancreatic insulin secretion thus we evaluated the plasmatic insulin levels and the insulin pancreatic content. However, the values obtained were below the detection limit of the kit so, no conclusion can be taken

from these particular experiments. An increase in the insulin pancreatic content was reported to exist only in long-term studies, which may explain these results since we only made a single dose administration (Moritoh *et al*., 2008, 2009; Reimer *et al*., 2002; van Genugten *et al*., 2012; Wu *et al*., 2012). Moreover, the animal number used to perform these experiments (n = 3 per group) was small considering the huge inherent variations of this disease.

Thus, no concrete conclusion can be taken with the results obtained and assuming that the PSi composite systems did not work seems very hasty. A study using a higher number of animals per group and for a longer period of time (chronic study) would be needed to have reliable results and know if the PSi composite systems are or not promising candidates to use as a therapy for T2DM.

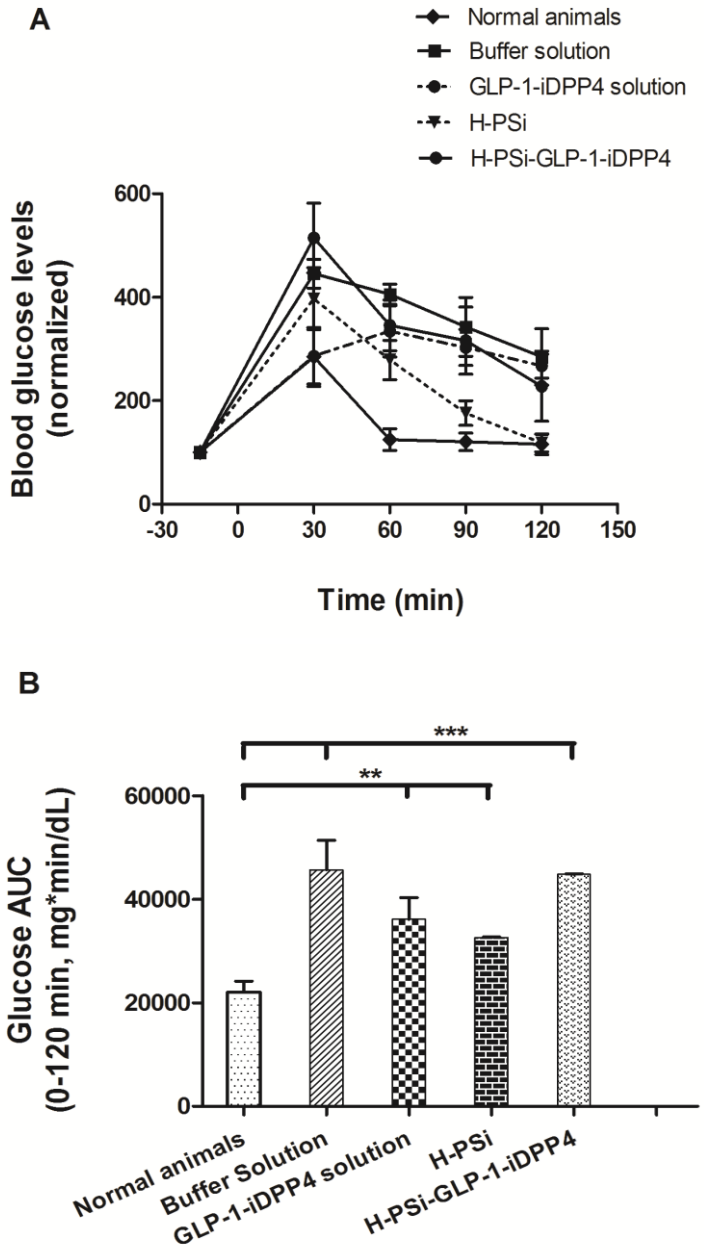

Figure A1. (**A**) Blood glucose levels of T2DM induced rats following i.p. administration of a glucose solution (2 g/kg). The values were normalized by the normal animals group. Results are presented as mean ± SD (n = 3). (**B**) Blood glucose AUC in the period of -15–120 min after glucose administration. Results are presented as mean ± SD (n = 3). The levels of significance were set at probabilities of $^{**}p < 0.01$, and $^{***}p < 0.001$, as compared with normal animals group.

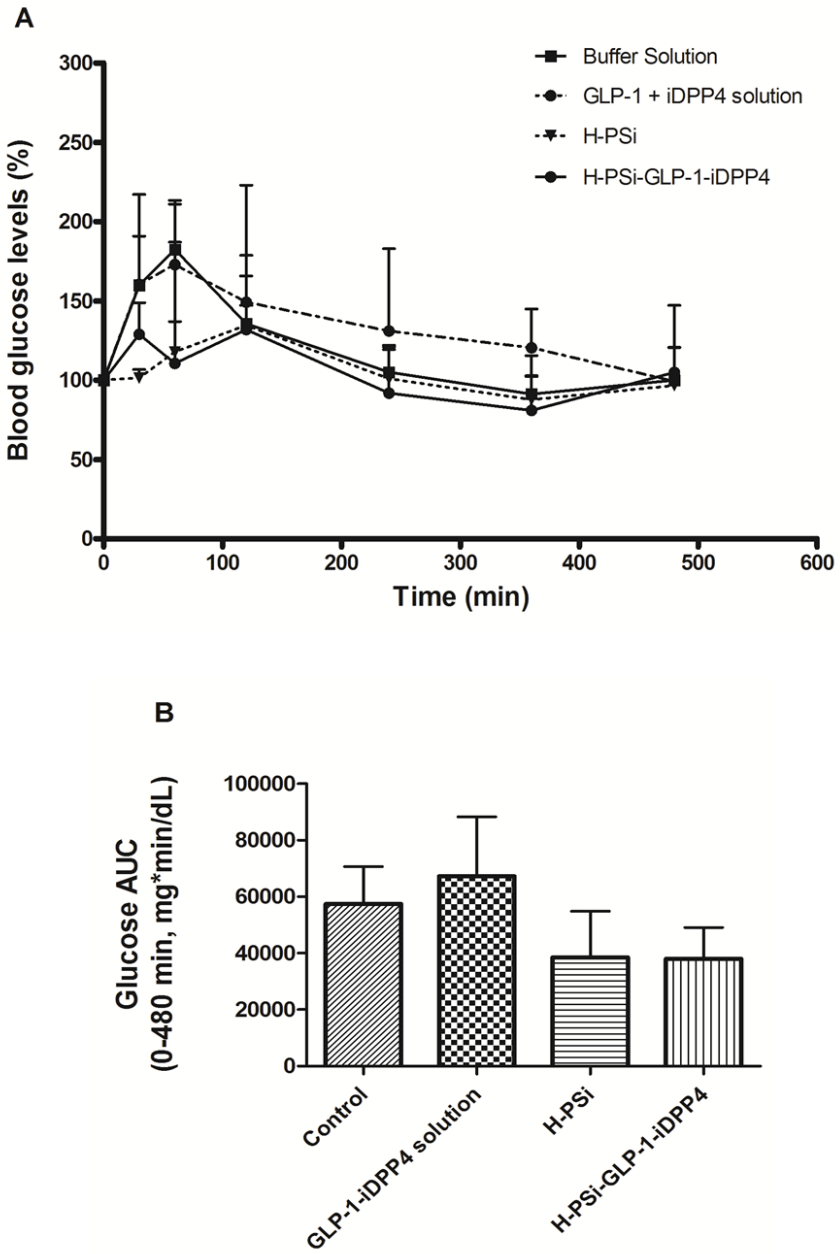

Figure A2. (**A**) Blood glucose levels of T2DM induced rats following oral administration of buffer solution (control), GLP-1-iDPP4 solution, H-PSi particles and H-PLGA-GLP-1-iDPP4 particles. Results are presented as mean ± SD (n = 3). (**B**) Blood glucose AUC in the period of 0– 480 min after oral administration. Results are presented as mean ± SD (n = 3).

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